Canella, Karen

From: STIC-DOCS
Sent: Wednesday, January 31, 2007 12:36 PM
To: Canella, Karen
Subject: ILL REQUEST (616705) 10727432

Your Request Number: 21758162
for "Cholesterol requirement for growth of IR983F and P" from CYTOBIOS
Relais Request Number: REG-14025634

http://public.nlm.nih.gov/projects/relais/6499033.pdf

This document contains 9 pages (including the cover page), is 1,341KB, was posted on January 31, 2007 at 10:15:05 AM and will be available for 7 days.

ILL Document Delivery

REG-14025632

VAUZGE

NLM -- W1 HY26 (GENCOLL)

US PATENT AND TRADEMARK OFFICE
SCIENTIFIC AND TECHNICAL INFO CTR
107 S. WEST STREET, PMB 803
ALEXANDRIA, VA 22314

ATTN:
PHONE: 571-272-2550
FAX: 571-272-0230
E-MAIL: STIC-DOCS@uspto.gov

SUBMITTED: 2007-01-30 15:21:51
PRINTED: 2007-01-31 09:44:49
REQUEST NO.: REG-14025632
SENT VIA: DOCLINE
DOCLINE NO: 21758186

REG **Copy** **Journal**

TITLE:	HYBRIDOMA AND HYBRIDOMICS
PUBLISHER/PLACE:	Mary Ann Liebert, Inc., Larchmont, NY :
VOLUME/ISSUE/PAGES:	2003 Aug;22(4):267-72 267-72
DATE:	2003
AUTHOR OF ARTICLE:	Ozturk S;Kaseko G;Mahaworasilpa T;Coster H
TITLE OF ARTICLE:	Adaptation of cell lines to serum-free culture med
ISSN:	1536-8599
OTHER NUMBERS/LETTERS:	
	NLM Unique ID: 101131136
	PubMed ID: 14511573
SOURCE:	PubMed
MAX COST:	$4.00
COPYRIGHT COMP.:	Guidelines
CALL NUMBER:	W1 HY26 (GENCOLL)
REQUESTER INFO:	616704
DELIVERY:	E-mail Post to Web: STIC-DOCS@uspto.gov
REPLY:	Mail:

KEEP THIS RECEIPT TO RECONCILE WITH BILLING STATEMENT

For problems or questions, contact NLM at http://wwwcf.nlm.nih.gov/ill/ill_web_form.cfm or phone 301-496-5511.

Include LIBID and request number.

HYBRIDOMA AND HYBRIDOMICS
Volume 22, Number 4, 2003

Short Communication

Adaptation of Cell Lines to Serum-Free Culture Medium

S. OZTURK,[1] G. KASEKO,[2] T. MAHAWORASILPA,[2] and H.G.L. COSTER[2]

ABSTRACT

The optimum conditions to allow proliferation of cells for the secretion of some growth factors and cytokines and the proliferation of cells in different media that do not contain proteins or serum from animals (serum-free media) were investigated. The culture of cell lines for the commercial production of products involves optimisation of cell proliferation and secretion in media from which the requisite proteins can be economically extracted. Some of these problems were addressed in this study. We used two different clones from a human myeloma cell line for adaptation to serum free medium in order to characterize long-term effects of the new medium. We gradually decreased serum content of medium and the results showed that cell mortality increased with serum reduction, antibody production lost by survived clones, and secretion of cytokines were always retained.

INTRODUCTION

SINCE THE INTRODUCTION OF HYBRIDOMA TECHNOLOGY by Köhler and Milstein, monoclonal antibodies (MAb) have been used extensively in clinical diagnostic, therapy, and purification of biomolecules. Optimal growth of hybridoma cells is routinely achieved in a defined basal medium containing relatively high levels of serum or protein supplements. To manufacture highly purified monoclonal antibodies of constant quality, a well-defined production process is necessary. Both serum and purified proteins should be omitted if possible. The use of chemically defined protein-free cell culture media offers the advantages of lot-to-lot consistency and reduced production costs. Furthermore, it facilitates downstream processing as well as regulatory approval and the biosafety of the product is improved.[1]

It has taken almost 25 years for MAbs to begin to realize their promised commercial potential. While murine (mouse-derived) monoclonal antibodies have good utility as research reagents and in diagnostic applications, they have had very limited success as therapeutics. One of the main reasons for the failure of many of the early therapeutic applications of murine antibodies was the production of human anti-mouse antibodies (HAMA), where the murine antibody is seen as foreign by the human host, who makes antibodies to this foreign protein. This side effect seriously reduced the effectiveness and safety of many murine monoclonal antibodies in clinical trials.[2]

Usually stable human hybridomas have been obtained only after extensive subcloning in media containing fetal bovine serum (FBS) and the purification of human MAb from FBS containing culture supernatant is demanding. Furthermore, the adaptation of cell lines to serum-free conditions by gradually reducing the content of FBS in culture medium, though possible, is time consuming and labor intensive.[3]

Investigation of the effects of growth factors and inhibitors on the proliferation of cancer cell lines involves the culturing of the cell lines in the presence of various growth factors and characterization of the response as a function of concentration of the growth factors. A parallel study would look at the effects of growth inhibitors and any cross correlation between these and the growth stimulators.

Hybridomas and myelomas that we used as a fusion partner are similar. Their cell origins are B lymphocyte. Neither is clonally stable. They can secrete MAb. Immortalization of hybridomas can be controlled but myelomas are uncontrolled. Specificity of MAb of hybridoma is known, but myeloma is unknown. Therefore, we used two clones of a human myeloma cell line (TOM H and TOM L) as model cell lines.

[1]Research Institute for Genetic Engineering and Biotechnology, Tübitak Marmara Research Center Gebze-Kocaeli, Turkey.
[2]The UNESCO Center for Membrane Science and Technology, The University of New South Wales, Sydney, Australia.

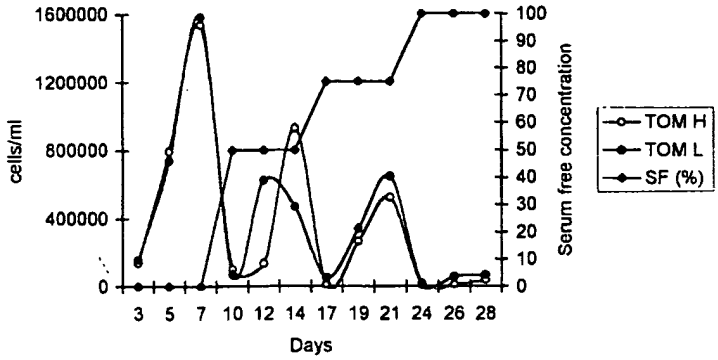


FIG. 1. Number of survived cells of TOM H and TOM L under different culture conditions.The serum content is expressed in terms relative to standard concentration of serum supplement used (10% FCS represents 0% serum free [SF], 5% FCS–50% SF, 2.5% FCS–75% SF, and 0% FCS is shown as 100% SF).

MATERIALS AND METHODS

The cells were routinely grown in a complete medium, that is, RPMI 1640 supplemented with 10% fetal calf serum, 20 mM Hepes, 1% $NaHCO_3$, and 4 mM L-glutamine. For all experiments, exponentially growing cells from the working cell bank, cultured in the complete medium at 37°C in a humidified 5% CO_2 atmosphere, were used. The effects of serum starvation on cell proliferation, antibody production, and secretion of growth factors were assayed in a chemically defined serum free medium (EX-CELL 610 HSF) by gradually decreasing the content of serum supplement. EX-CELL 610 HSF was purchased from JRH Biosciences. It contained insulin, transferrin, sodium selenite, 2-mercaptoethanol, 2-aminoethanol, sodium pyruvate, glutamine, and bovine serum albumin. The human sub-lines TOM H and TOM L secreting IgM at different concentrations (0.4 and 0.12 mg/mL, respectively) were derived from single cell clones of human myeloma cell line (ATCC CL 187). For the experiments, the cells were seeded in 25 cm^2 flasks at a cell density of 1×10^4 cells/mL in media containing different concentrations of serum and compared with a control, RPMI 1640 containing 10% FCS. Tissue cultureware for cell culture and ELISA was supplied by Edward Keller. The cells were harvested by centrifugation on the third, fifth, and seventh days and the supernanatants were analyzed by ELISA for concentrations of IgM, human tumor necrosis factor-α (TNF-α), granulocyte macrophage–colony stimulating factor (GM-CSF), and interleukin 10 (IL 10). In order to determine the amount of IgM secreted by the cells, sandwich ELISAs were performed in 96-well flat bottom EIA/RIA plates (certified) coated with goat anti-human IgM (μ chain specific) (supplied by ICN cat no. 1-61021). Coating of the plates was carried out at 4°C overnight. The plates were washed three times with a wash buffer (0.005% Tween 20 in PBS), then 5% skim milk powder (w/v) in PBS-T (0.005% Tween 20 in PBS) was added to the wells, and the plates were incubated for 1 h at 37°C followed by washing as mentioned before. Fifty milliliters of serially diluted standard (human IgM, supplied by ICN catalog no. 65345) or sample were added to the wells. The plates were incubated at 37°C for 1 h then washed three times with the wash buffer. Goat anti-human IgM-HRP conjugate (supplied by ICN catalog no. 68-392) was diluted 1:3000 with PBS and pipetted 50 mL per well. The plates were incubated at 37°C for 1 h and then washed three times with the wash buffer. A substrate solution was prepared by dissolving 0.0066 g of ABTS in 6 mL of citric acid/phosphate buffer and then adding 3 mL of 30% H_2O_2 to the solution. Fifty milliliters of the substrate was added to each well. The plates were incubated at room temperature until the

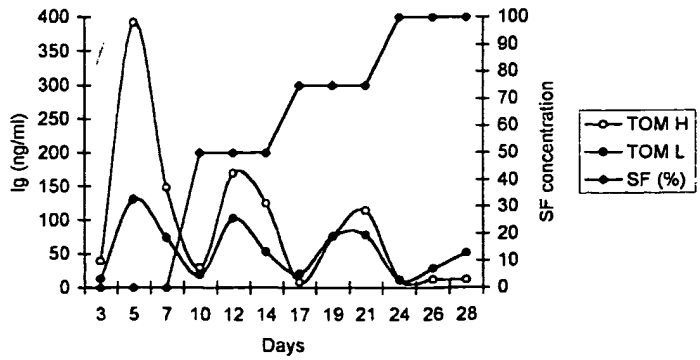


FIG. 2. Production of IgM by TOM H and TOM L in medium with different content of serum.

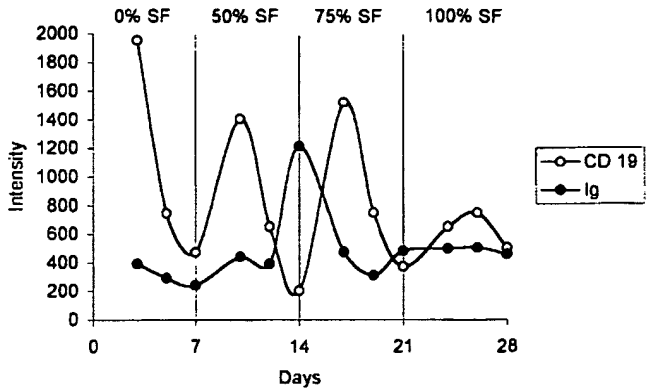


FIG. 3. Analysis of changes in surface expression of CD 19 and sIg by TOM H. Cells were grown in 0% SF up to day 7; from day 8 to day 14 in 50% SF; from day 15 to day 22 in 75% SF; from day 23 to 28 in 100% SF.

most concentrated sample had A_{405} of 1.0. The reaction was stopped with 50ml of saturated NaF. Quantitative Immunoassay Kits (Qantikine) were used to determine the concentrations of TNF-α, GM-CSF and IL 10 in the cell culture supernatants. These assays employ the quantative sandwich enzyme immunoassay technique where monoclonal antibodies specific for TNF-α, GM-CSF and IL 10 have been precoated onto a microplate. The assays were carried out according to the manufacturer's instructions. Following every viability and cell growth counting, phenotypic analyses of the surface immunoglobulin and CD 19 expressed by the cells grown in the medium with different serum contents, were performed with monoclonal antibodies specific to human Ig and human CD 19 and analyzed by FACS Vantage SE (Beckman). For these analyses, the cells were harvested and washed with PBS. The cell concentration in each analyzed sample was adjusted to 10^6 cells/mL. One hundred milliliters of each sample was incubated with 5 mL of mouse anti-human CD 19 conjugated with PE or mouse anti-human Ig conjugated with FITC according to the supplier's instructions, on ice in the dark for 15 min. Then the cells were washed three times with PBS in order to remove unbound antibodies and suspended in 100 mL of PBS for further analysis. The standard of human IgM, anti-human IgM, anti-human Ig-FITC, anti-human CD 19–PE, and anti-human Ig-peroxidase conjugates were purchased from SIGMA.

RESULTS

Cell proliferation in the medium with different serum contents

There is no difference in survival between TOM H and TOM L cultures under standard conditions (Fig. 1). However, the cell mortality increased with reduction of serum supplement for both sub-lines.

Production of IgM in the medium with different serum contents

TOM H lost half of the antibody production when it was cultured in only 50% serum free whereas TOM L was able to maintain the production of IgM almost at the original level. In 100% serum-free environment TOM L produced IgM four times higher than that secreted by TOM H, which made TOM L a higher antibody producer under serum free conditions compared to TOM H (Fig. 2).

Maintenance of CD 19 and surface Ig expression in the medium with different serum contents on day 5 of adaptation

Approximately 20% of cells in both TOM H and TOM L cultures lost the expression of CD19 by the 5th day when adapted to 100% serum free conditions. However, the sharp de-

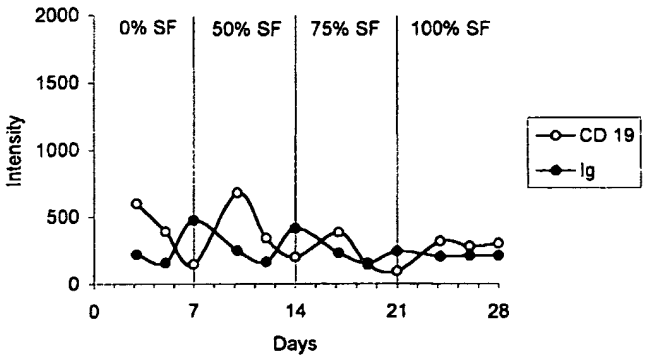


FIG. 4. Analysis of changes in surface expression of CD 19 and sIg by TOM L

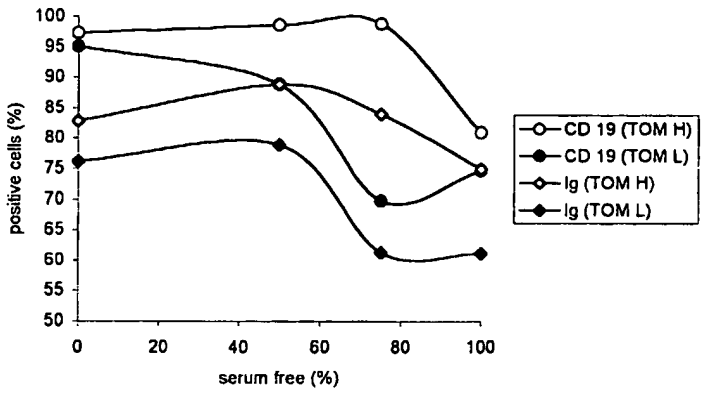


FIG. 5. Number of cells expressing CD 19 and sIg in TOM H and TOM L on the 5th day of cultures in different serum contents of the culture medium.

cline in the numbers of CD19-positive cells in TOM H was observed after achieving 75% SF, whereas for TOM L the decline was smoother with crucial point at 50% SF. The higher expression of sIgM by TOM H on day 5 at different SF conditions directly correlated with higher IgM concentration in supernatants compared to TOM L (Figs. 3 and 4).

Changes in level of CD 19 and Ig expression during adaptation to serum free conditions

A gradual decrease of serum content resulted in a diminished expression of CD19 during serum free adoptation process. Percentage of CD19-positive cells in both TOM H and TOM L also decreased. Expression of sIgM changed in a similar fashion to CD19 expression. We also observed that the density of CD19 expression on cell surface under all serum free culture conditions always reached its maximum at the end of exponential growth of the culture followed by a sharp decline at the confluence. Noticeably, TOM L had less prominent changes in level of CD19 expression.

The number of cells expressing CD19 and sIg in TOM H and TOM L on the 5th day of cultures in different culture medium are shown in Fig. 5.

Production of GM-CSF, TNF-α, and IL-10 by TOM H and TOM L in the medium with different serum contents

Both TOM H and TOM L were able to maintain the production of growth factors up to 75% SF followed by a sharp decline corresponding to the decrease of CD19 and sIgM expression with the exception of GM-CSF secreted by TOM L culture. In 0% SF environment, TOM L secreted GM-CSF at levels higher than when transferred to 50% and 75% SF environments. Also, TOM L secreted higher levels of GM-CSF in a reduced serum condition compared to TOM H, which correlated with higher concentrations of IgM secreted into culture supernatants (Fig. 5). The changes in the production after growth factors with reduction of serum supplement for TOM H and TOM L and the level of GM-CSF and IL-10 secretion by TOM H and TOM L at different medium conditions on day 7 of culture are shown in Figs. 6 and 7, respectively.

DISCUSSION

The success of MAb production for therapeutics, prophylactics or diagnostics is largely dependent on the ability of hybridomas to maintain their Ab production during culturing and

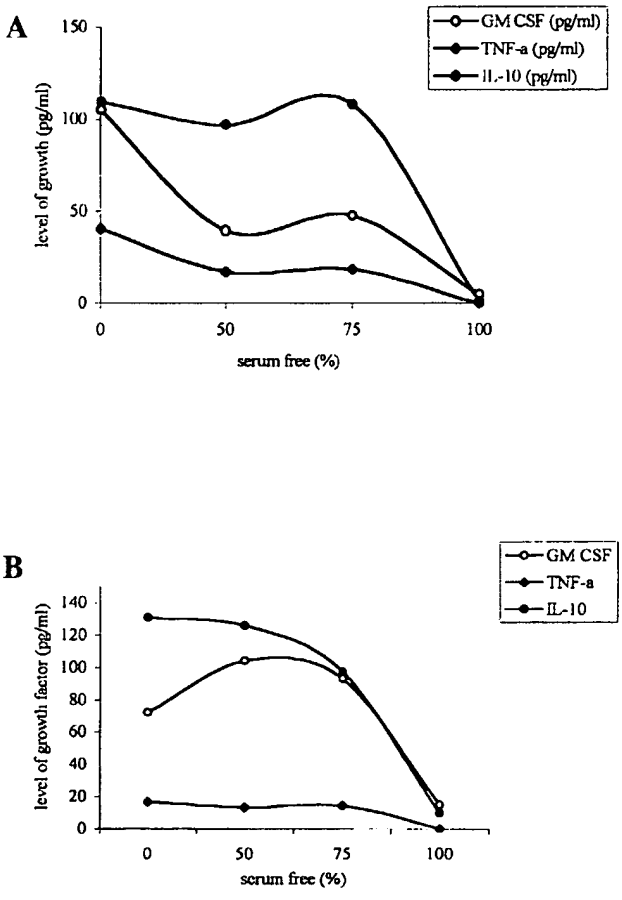


FIG. 6. Changes in the production of the growth factors with reduction of serum supplement. (**A**) TOM H. (**B**) TOM L.

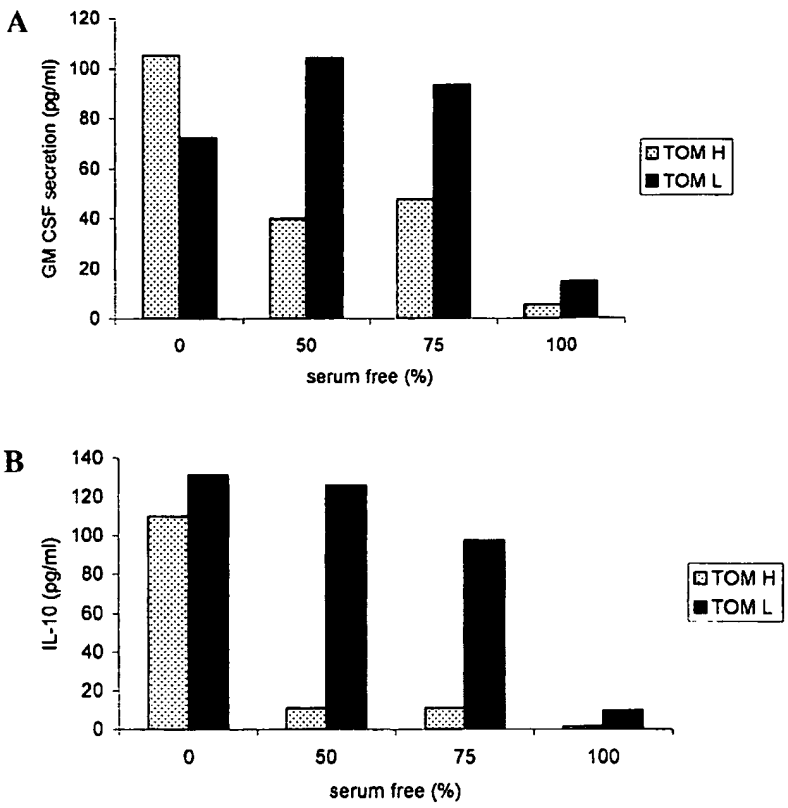


FIG. 7. Level of GM-CSF (A) and IL-10 (B) secretion by TOM H and TOM L at different medium conditions on day 7 of culture.

adaptation to serum-free conditions. Serum-free culture is used routinely for many cell types (e.g., CHO, hybridomas, and recombinant myeloma cell lines). Serum free medium reduces operating costs and process variability, and removes a potential source of infectious agents.[4,5]

In this study, we tried to characterize two cell sub-lines derived from single cell clones of the same parental myeloma cell line, based on the different levels of their antibody production. The sub-lines were grown in the cultures with various contents of serum supplement. We wanted to see the effect of gradual serum withdrawal on the proliferation, differentiation and secretion of these two sub-lines. For this purpose, we investigated the importance of maintaining the autocrine secretion of growth factors for the proliferation and Ig production of these sub-lines, as well as CD19 and Ig expressions. The observation for each culture condition points were taken on days 3, 5, and 7. We monitored the cell concentrations and collected supernatants for ELISA, electrophoresis, and HPLC analysis. We also collected cell samples from cultures with different contents of serum supplement for FACS analysis (CD19 and Ig expression analysis) on days 3, 5, and 7. CD19 is a central response regulator of B lymphocyte signaling thresholds governing autoimmunity. The CD19/CD21 complex is categorized among the "response regulator" class of receptors, which determine the magnitude and outcomes of B cell receptor signals. Small changes in CD19 expression have dramatic effects on signaling thresholds within B cells. We found that CD19 expression declined with the reduction of serum supplement and correlated with the ability of sub-lines to produce Ig in a serum free environment. TOM L, which produced less IgM in the medium supplemented with 10% FCS, retained higher levels of CD19 expression and secreted more IgM in the medium free of FCS compared to TOM H. Also, the level of CD19 expression by both sub-lines was cell growth dependent. The maximum of CD19 expression was observed at the stage of exponential growth followed by a sharp decline at the point of culture confluence.[6,7]

GM-CSF, which is a pleiotropic cytokine, can stimulate the proliferation, maturation, and function of cells of the immune system. It is produced by a variety of cell types: macrophages, mast cells, endothelial cells, fibroblasts, T and B lymphocytes in response to cytokine or immune and inflammatory stimuli. In tumor cell lines, the rate of cell proliferation was correlated with expression of GM-CSF receptors. The addition of anti-GM CSF monoclonal antibody to a culture of lymphocyte inhibits the lymphocyte proliferation. Our findings suggest that there is a positive correlation between the levels of CD19 and sIgM expressions by sub-lines and the maintenance of GM-CSF secretion. The higher levels of GM-CSF secretion, the higher levels of CD19 expression and consequently IgM production were observed. TOM L had greater ability to produce GM-CSF and maintain or even increase the antibody yield when grown in a serum free environment. We investigated (data not presented) the presence of autocrine growth regulation and found that TOM L sub-line expressed GM-CSF receptor (GM-CSF-R) whereas cells of TOM H sub-line lacked the expression of GM-CSF-R.TNF-α is produced by neutrophils, activated T and B lymphocytes, NK cells, LAK cells, astrocytes, endothelial cells,

smooth muscle cells and some transformed cells. TNF-α has lytic activity and may also have an important role in intra-cellular communication. Overproduction of TNFs has been implicated as playing a role in a number of pathological conditions. Even through there was a general decline in TNF-α production by both sub-lines, there was no striking correlation with the levels of IgM production or CD19 and sIg expressions.

IL-10 is mainly produced by mononuclear cells and possesses a wide range of activities on a number of cell types, including monocytes, T and B lymphocytes, NK cells, dendritic cells. Ig production is enhanced by IL-10. IL-10 also functions as a differentiation factor for plasma cells. IL-10 has been shown to profoundly inhibit gene expression and production of numerous cytokines, including IL-1α, Il-1β, IL-3, IL-6, IL-8, TNF-α, G-CSF, and GM-CSF in monocytes and T lymphocytes. The most striking activity of IL-10 is a powerful effect on the stimulation of Ig (IgG, IgM, and IgA) secretion in several B cell activation systems. The proliferation of memory B cells was interrupted by addition of IL-10 which induced the differentiation into plasma cells. Our study showed that levels of IL-10 secretion were not greatly affected by the reduction of serum content and did not correlate with the ability of sub-lines to retain the production of antibodies.[8,9]

The serum-free adaptation improves the effective purification of MAb. It also reduces operating costs, process variability and removes a potential source of infectious agents, meeting strict regulatory requirements for the manufacture of human therapeutic and diagnostic products. Consequently, there is a need for effective methods to adopt hybridomas to a serum free environment. However, there is no universal serum free medium which is suitable to every hybridoma. Therefore, the successful adaptation of one hybridoma does not guarantee that it works for others. It is known that there are controversial reports of serum-free effects on antibody production.[10] The stability of the specific production rate of hybridomas is dependent on the cell line and the culture medium. High cell viability can be maintained and in some cases an increased yield of monoclonal antibody can be observed in the protein or serum-free medium. Some investigators report difficulty in adapting hybridomas that produce IgM or IgA antibodies to serum free conditions.[11] The reason for emphasis on IgM production is that IgM is a potent complement-fixing antibody generated early in the human immune response in many infectious diseases. These observations indicate the need for production of IgM isotypes that are biologically active and exibit high affinity. Our study showed that expression of surface markers such as CD19 and sIg indicated the suitability of a particular cell culture medium or culture conditions for the maintenance of IgM production. This was especially important in a view that the initial levels of Ig production were not indicative of final yields of IgM in 100% serum-free environment. The lower secreting sub-line TOM L produced higher levels of IgM in the serum free medium compared to its initially higher secreting counterpart TOM H. Because both sub-lines originated from the same parental myeloma, our findings show that antibody secreting cell lines contain cell sub-populations with different abilities to survive and maintain the antibody production in the serum free environment. Thus, the assessment of autocrine regulation and expressions of CD19 and sIg provides a quick and efficient method to assess the effects of serum free environment on antibody production. Also, the presence of autocrine regulation through GM-CSF and corresponding GM-CSF surface receptor plays a crucial role in maintenance of IgM production.

ACKNOWLEDGMENT

We would like to thank the Research Grant Committee of the UNESCO Centre for Membrane Science and Technology, UNSW, Sydney, Australia.

REFERENCES

1. Ohlin M: *Human Monoclonal Antibody Technology. A tool to investigate human antibody repertoires.* Grahns Boktryckeri, Lund, Sweden, 1992.
2. Dahlenborg K: *Cellular and Molecular Aspects of the Germinal Centre Reaction.* Bloms i Lund Tryckeri AB, Lund, Sweden, 1998.
3. Lang AB, Schürch U, Zimmermann F, Bruderer U: Selective generation of antigen-specific human hybridomas optimized for large scale growth in serum free medium. J Immunol Methods 1992;154: 21–26.
4. Froud SJ: The development, benefit and disadvantages of serum-free media. Dev Biol Stand 1999;99:157–166.
5. Liu C, and Hong L: Development of shaking bioreactor system for animal cell cultures. Biochem Eng J 2001;7:121–125.
6. Ling NR: Immunoglobulin production by cultured human lymphocytes. J Immunol Methods 2000;238:3–15.
7. Sato S: CD 19 is a central response regulator of B lymphocyte signaling thresholds governing autoimmunity. J Dermatol Sci 1999; 22:1–10.
8. Nagumo H, and Agematsu K: Synergetic augmentative effect of IL-10 and CD 27/CD 70 interactions on B-cell immunoglobulin synthesis. 1998;94:388–394.
9. Choe J, and Choi YS: IL-10 interrupt memory B cell expansion in the germinal center by inducing differentiation into plasma cells. Eur J Immunol 1998;28:508–515.
10. Ghosh R, and Cui ZF: Protein purification by ultrafiltration with pre-treated membrane. J Membr Sci 2000;167:47–53.
11. Ward PA, Adams J, Faustman D, *et al.: Monoclonal Antibody Production: A Report of the Committee on Methods of Producing Monoclonal Antibodies.* Institute for Laboratory Animal Research, National Research Council, National Academy Press, Washington, DC, 1999.

Address reprint requests to:
S. Ozturk, Ph.D.
Research Institute for Genetic Engineering and Biotechnology
Tübitak Marmara Research Center
41470 Gebze-Kocaeli, Turkey

E-mail: selma@rigeb.gov.tr

Received for publication May 5, 2003. Accepted for publication June 10, 2003.

AN AFFINITY BASED ISOLATION PROCESS FOR MYELOMA tPA

James Callaway, Ph. D.

Department of Biological Process Sciences
SmithKline French Res. Labs
SmithKline Beecham
King of Prussia, PA 19406

Collaborators

D.C. Greene, R.D. Sitrin, M.B. O'Hara, A. Patel, A.H. Nishikawa

Abstract

An isolation process was developed for a tissue plasminogen activator (tPA) possessing the same primary sequence as the wild-type form. The molecule was expressed in a myeloma cell line and secreted into a serum-free medium using a perfusion system with a production cycle approaching one hundred days. The tPA was recovered as 76-90%. single chain but lacked detectable levels of sialic acid and possessed significantly reduced solubility near neutral pH as compared to CHO derived tPA. The isolation process employs a Metal Chelate Affinity capture step followed by neutral pH precipitation. A tripeptide based affinity step is utilized with a high ionic strength binding and wash conditions to remove trace contaminants. Size exclusion chromatography is used as a final polishing step. The process is designed to meet regulatory requirements for clearance of DNA and adventitious agents, incorporates viral kill steps, and produces a product with a protein purity of greater than ninety-five percent. The isolation process and final product characterization for this myeloma tPA is compared to the tPA derived from a CHO cell line used in clinical studies.

Biography

James Callaway is a Sr. Investigator within the Bioseparations group of the Department of Biological Process Sciences at SmithKline and French Res. Laboratories. He holds a B.A. degree in chemistry from Calif. State Univ., Chico and a Ph.D. in biological chemistry from UCLA. Dr. Callaway is currently involved in the production of the soluble CD4 receptor at SmithKline and French for Phase II clinical studies.

INTRODUCTION:

Tissue-type plasminogen activator (tPA) is a serine protease which acts at the fibrin surface to convert plasminogen to plasin, thereby facilitating fibrinolysis (1). The glycoprotein exists as a single polypeptide chain with a molecular weight of 70,000 daltons, which upon proteolysis is converted to two polypeptides of 32-36,000 daltons linked through a single disulfide bond (2,3).

The goal at SmithKline and French was to identify a unique expression system for tPA production. A myeloma cell line was selected due in part to the extensive characterization of this expression system as an antibody production vehicle. The tPA gene was introduced into the myeloma cell line and amplified. A high producing clone was isolated and placed in a perfusion style cell culture production system. What resulted was the production of a unique form of tPA, lacking sialic acid in the N-linked carbohydrate structure. This paper discusses the adaptation of the isolation process to accommodate the cell culture production system and the unique physical properties associated with the lack of sialic acid.

MATERIALS AND METHODS:

Cell Culture

The media used for the production phase of the perfusion cell culture system was composed of 2% BSA, transferrin, basal media and Excyte at 2 ml/L [a complex lipid supplement isolated from bovine serum]. The production system used four liters of packed cell matrix perfused at a rate of approximately 25 liters per day.

Metal Chelate Affinity

Chelating Sepharose Fast Flow from Pharmacia was used for the Metal Chelate Affinity resin, as introduced by J. Porath (4). A 3-6 L column was employed in a column with 500 cm^2 of cross sectional area. Media loading and all wash steps were performed at 4^oC at 240 cm/hr, elution was affected at 120 cm/hr to keep the eluate volume small. $ZnCl_2$ was used to charge the resin and was added to the media at 10 ppm prior to loading. Following loading the column was washed with 5 column volumes of 0.1 M NH_4AC/pH 6 and then with 2-3 column volumes of 0.1 M NH_4AC/0.1 M NaCl/pH 4.5. Elution was affected by 0.1 M NH_4AC/0.5 M NaCl/pH 4.5. The column capacity for the crude tPA was approximately 2 grams per liter resin.

pH 7 Precipitation

The zinc chelate eluate was adjusted to pH 7 with 1 N NaOH and the mixture was stirred for 30 min at 4 ^oC, the precipitate is then collected by centrifugation at 5,000 x g. The pellet was resuspended by the addition of 0.1 M acetic acid. Following solubilization the material was adjusted to 300 mM NaCl with 600 mM NaCl/0.1 M NH_4AC/pH 4.

Transition State Analog

The tripeptide was built onto a Sepharose-CL6B resin activated by epibromohydrin chemistry. Ethylenediamine was used to provided a hydrophilic linker which was attached to succinic anhydride. Amino acids were placed on as either a tripeptide or in a sequential manner by condensation with carbodiimide. The argininial form was produced from the arginine semicarbazone with formaldehyde.

The resin was packed into 5x21 cm column and operated at 150 cm/hr at ambient temperature. The resuspended pellet was loaded and the column was washed with 5 volumes of 0.1 M $NaPO_4$/pH 7 and 1 M NaCl/0.1 M $NaPO_4$/pH 7. The salt was then reduced by 5 volumes of 0.1 M $NaPO_4$/pH 7 prior to elution with 0.1 M acetic acid.

Superose-12 Chromatography

The eluate from the transition state analog column was concentrated on a tangential flow apparatus using 10 kD nominal molecular weight cutoff membranes. Sodium chloride was added to bring the salt concentration to 200 mM. The column (11.3x90 cm) was equilibrated and run at 20 cm/hr with 200 mM NaCl/100 mM HOAc at room temperature.

RESULTS:

Metal Chelate Affinity

The tPA titer in the conditioned media varied throughout the course of the production cycle, being highest in the 20-40 day period. The titers were routinely below 10 mg/L, which required the passing of large volumes of conditioned medium through the capture column. It was observed that when the application exceeded 300 L of conditioned media per liter of resin, tPA began appearing in the flow through even though the capacity of 2 grams of tPA per liter of resin was not surpassed. As tPA began appearing in the flow through the level of zinc ions also increased indicating that a media component was striping the zinc ions from the column, reducing the binding capacity of the resin. The addition of $ZnCl_2$ to the conditioned media prior to chromatography at a level 10 ppm allowed for loading of conditioned media to levels of greater than 1,500 L per liter of resin.

The vast majority of media components pass through the column without being retained, while the tPA is quantitatively retained. The wash at pH 6 removes a large percentage of the residual bovine serum albumin (BSA). The pH is lowered to 4.5 in the presence of 100 mM NaCl; upon inspection with SDS-PAGE this wash contains a heterogeneous population of proteins. tPA will begin to slowly elute if more than three column volumes of this buffer are applied to the column. The product is eluted at in 500 mM NaCl at pH 4.5. These elution conditions are distinctly different from those originally worked out for tPA (3), which operated at pH 7 and affected elution with a histidine gradient. Low pH elution is another classical method of elution of proteins from Metal Chelate Chromatography columns (5) and affords better solubility for the myeloma tPA.

The yield for this step is approximately 97% as determined by the amidolytic S-2251 assay and affords a 10-fold reduction in volume. The material ranges from 85-89% tPA as judged by SDS-PAGE under nonreducing conditions after this single step which represents an 18,000 fold enrichment. The Metal Chelate Affinity chromatography step has been successful in producing a 4 log reductions of DNA as a capture step from conditioned media.

pH 7 Precipitation

The zinc chelate eluate is adjusted to pH 7 with 1 N NaOH and the mixture is stirred for 30 min at 4 ^{o}C, the precipitate is then collected by centrifugation at 5,000 x g. The trace levels of bovine serum albumin remain in the supernatant, affecting a 1-2 log reduction in BSA levels. The pellet is firm and is readily resuspended in low ionic strength buffer affording a recovery of 95-97%. Alternatively, the pellet can be stored for many months at -70^{o}C to provide for stock-piling of inprocess material for consolidation of several capture steps. This also provides shipment of the product from the off site cell culture vendor to the final processing site.

Transition-State Analog Chromatography

This column was designed to provide column selectivity on the basis of the plasminogen binding site of tPA. The binding of tPA to the tripeptide column is driven by a high ionic strength environment. The resuspended pH 7 precipitate was adjusted to 0.3 M NaCl and applied to the column. The pH of the column was then adjusted to 7 and washed with 1 M NaCl to remove the residual DNA and BSA. Elution was affected by lowering the ionic strength and pH with 0.1 M acetic acid. The eluate is held at this low pH to provide effective viral inactivation. The yield for this step is 95%, with the purity of the product routinely surpassed 95%.

Size Exclusion Chromatography

The final polishing step is size exclusion chromatography. The eluate from the Transition State Analog column is adjusted to 0.2 M NaCl and applied to a Superose-12 column. A peak migrating near the void of the column is determined to be tPA by SDS-PAGE under reducing conditions. The tail of the product peak is not pooled with the rest of the material as it contains low molecular weight contaminants. This step results in a 90% yield and is very effective in removing non-covalent aggregates of tPA which co-purify with the tPA through the affinity steps. DNA reduction is projected to be 2-3 logs depending on the size of the fragment. Buffer exchange is required to place the material into the appropriate buffer for delivery of Bulk Drug Substance [100 mM ammonium acetate/pH 4]. Attempts to perform the buffer exchange in the column was not successful because at the lower ionic strength the molecule interacted with the resin. A summary of the yield for each step of the process is presented in Table I. Each step results in a high percent recovery and the use of only three chromatographic steps results in a total recovery of 76%.

TABLE I
PROCESS RECOVERY SUMMARY

STEP	CONCENTRATION IU/ML (10^{-3})	VOLUME [L]	GRAMS	YIELD [%]
Conditioned Media	1.9	1,100	3.8	-
Zinc Chelate	147	13	3.6	97
PH 7 Precipitation	235	8	3.4	92
Trans State Anal	1,400	1.2	3.2	87
Superose-12 / Diafil.	3,150	0.47	2.8	76

Characterization

The molecule isolated from this myeloma cell line is compared to that of tPA derived from an attached CHO cell line in defined media (Table II). There are two striking differences between the tPA isolated from this myeloma cell line when compared to the tPA isolated from a CHO cell line. The first being the lack of sialic acid residues (NANA), which is assumed to account for the reduced solubility of this form of tPA. The second is that this tPA has a significantly higher level of single chain tPA. The latter is presumed to be related to the use of serum free media for production and that the perfusion system used in production of the myeloma tPA removes the protein from the proximity of the cells much more rapidly than that of the production system used for the attached CHO cells.

TABLE II
COMPARISON OF MYELOMA AND CHO TPA

PHYSICAL PROPERTY	MYELOMA TPA	CHO TPA
Purity (nonreducing SDS-PAGE)	95-97	95-98
Single Chain (%)	76-90	32-68
L-Chain Content (%)	29	1-18
Specific Activity (S-2251 [x10^5 IU/mg])	5.1-5.7	5.8-6.8
Carbohydrate (wt%)	6-7	8-12
Mannose (wt%)	8-9	9-12
Sialic acid [NANA] (wt%)	0-0.1	3-5

DISCUSSION:

The isolation of myeloma tPA is quite different from that developed at SKF for the production of clinical supplies of CHO derived tPA. The primary cause is that the tPA isolated from this myeloma cell line has distinctly different biophysical properties, namely the lack of sialic acid residues on the N-linked carbohydrate chains. The process was designed to take advantage of the low solubility of the myeloma tPA by the addition of the pH 7 precipitation step. In addition, all elution steps in the process are at pH 4 or below to avoid solubility problems inherent in the scale up of many processes, but which were exacerbated by the absence of sialic acid in this molecule. The process was made more efficient by implementing vigorous washing of each column, which allowed for a reduction in the number of steps while maintaining the level of purity.

Benzamidine was used as the active site directed affinity step for CHO derived tPA isolated at SKF. A more selective resin was developed for the myeloma tPA. The D-Phe-D-Phe-Arg (aldehyde form) peptide was identified by an exhaustive search of di- and tripeptide sequences, using both D and L amino acids. The peptides with the most efficient binding were those with one or two hydrophobic residues at the distal end of the tripeptide. Insertion of D amino acids was not required for binding, but inhibited proteolysis of the peptide on the resin. The approaches used in the design of this purification step may be useful in the design of processes of some of the carbohydrate mutants currently under study which are also projected to have reduced solubility.

Working downstream of a perfusion type reactor technology dictates that the process be forgiving at the capture step. The process must handle a variable range of product streams caused by the changes in product titer and the number of reactors on line at any point during the production run. Control of the input stream is critical to the optimization of any process step. Since perfusion type cell culture systems are in flux, this is not possible in the capture step. Therefore, it is valuable to design a stock-piling step to allow for the remaining steps in the process to handle a constant, predicable flow of product, allowing for optimum use of equipment, personnel, and chromatographic parameters. In addition, unforeseen but inevitable events which cause delays in downstream processing are more acute when a perfusion system is implemented in cell culture. This is due to the justified reluctance to slow down or suspend production since a major economic advantage to the perfusion approach is based on the continual production of material. By stock-piling material prior to downstream processing, the interdependent relationship between purification and cell culture production can be avoided in the critical final steps of the process.

The data presented illustrates the impact the cell line and cell culture production technology has on the biophysical characteristics of a recombinant molecule. These changes require an adaptation of the purification process. In this paper, affinity chromatography was utilized to optimize the yield, adapt to the limited solubility, and accept variability in the titer from the conditioned media.

REFERENCES

1. Robbins, K.C., Summaria, L., Hsieh, B., and Shah, R.J. (1967) **JBC** 242, 2333-2342.

2. Kok, P. and Astrup, T. (1969) **Biochemistry** 8, 79-86.

3. Rijeken, D.C., Collen, D. (1981) **JBC** 256, 1035-7041.

4. Porath, J., Carlsson, J., Olsson, I., Belfrage, G. (1975) **Nature** 258, 598-599.

5. Porath, J. (1985) **Modern Methods in Protein Chemistry,** Vol. 2, 85-95.

ILL Document Delivery

REG-14025634

VAUZGE

NLM -- W1 CY754 (GENCOLL)

US PATENT AND TRADEMARK OFFICE
SCIENTIFIC AND TECHNICAL INFO CTR
107 S. WEST STREET, PMB 803
ALEXANDRIA, VA 22314

ATTN:
PHONE: 571-272-2550
FAX: 571-272-0230
E-MAIL: STIC-DOCS@uspto.gov

SUBMITTED: 2007-01-30 15:20:59
PRINTED: 2007-01-31 10:15:01
REQUEST NO.: REG-14025634
SENT VIA: DOCLINE
DOCLINE NO: 21758162

REG **Copy** **Journal**

TITLE:	CYTOBIOS
PUBLISHER/PLACE:	Faculty Press. Cambridge, Eng.,
VOLUME/ISSUE/PAGES:	1991;68(272):15-22 15-22
DATE:	1991
AUTHOR OF ARTICLE:	Li JL;Li YJ;Chao YJ;Lin LX;Ouyang MH;Peng YB ;Chang WS
TITLE OF ARTICLE:	Cholesterol requirement for growth of IR983F and P
ISSN:	0011-4529
OTHER NUMBERS/LETTERS:	
	NLM Unique ID: 0207227
	PubMed ID: 1778091
SOURCE:	PubMed
MAX COST:	$4.00
COPYRIGHT COMP.:	Guidelines
CALL NUMBER:	W1 CY754 (GENCOLL)
REQUESTER INFO:	616705
DELIVERY:	E-mail Post to Web: STIC-DOCS@uspto.gov
REPLY:	Mail:

KEEP THIS RECEIPT TO RECONCILE WITH BILLING STATEMENT

For problems or questions, contact NLM at http://wwwcf.nlm.nih.gov/ill/ill_web_form.cfm or phone 301-496-5511.

Include LIBID and request number.

Cholesterol requirement for growth of IR983F and P3X63-Ag8-U1 myeloma cells in serum-free medium

Ji-Liang Li*, Ying-Jie Li, Sui Chao, Lai-Xing-Mei Lin, Ming-Hui Ouyang, Yi-Bing Peng and Wen-Sheng Chang

Department of Malaria Immunology, The First Medical University of PLA, Guangzhou, GD 510515, The People's Republic of China (*Reprint address)

Abstract

Cholesterol, a major lipid component of the plasma membrane, is thought to have profound effects on the structure and function of cells. Most animal tissues are capable of synthesizing cholesterol *de novo* from acetate; however, there are relatively few mammalian cells *in vitro* expressing an absolute requirement for an exogenous source of cholesterol. In this paper, it was shown that both IR983F (983) rat myeloma cells and P3X63-Ag8-U1 (P3U1) mouse myeloma cells which had been cultivated in serum-free medium containing cholesterol for more than 6 months still required cholesterol *in vitro* for growth in serum-free medium. Optimal growth of 983 and P3U1 occurred in cholesterol concentrations of 15 and 5 μg/ml, respectively. Moreover, it was demonstrated that the cholesterol could be replaced by human low density lipoprotein in a concentration of 10 μg/ml but not by mevalonic acid lactone. In contrast to the parental myeloma cells, hybridoma cells derived from the mouse myeloma cells which had been cultivated in serum-free medium containing cholesterol for more than 6 months did not require cholesterol.

Introduction

Cholesterol, a major lipid component of plasma membranes (Lange and Ramos, 1983), has profound effects on the structure and function of cells (Chen and Kandutsch, 1981). Evidence from mycoplasma and mammalian cells has shown that cholesterol levels affect membrane fluidity (Kroes *et al.*, 1972; Rottem *et al.*, 1973a,b) and permeability (Bruckdorfer *et al.*, 1969; DeKruyff *et al.*, 1972). Cholesterol content also influences the ability of cells to transport monovalent cations (Kroes and Ostwald, 1971; Poznansky *et al.*, 1973), to undergo endocytosis (Heiniger *et al.*, 1976; Sodergren *et al.*, 1983) and to express membrane-associated enzyme activity (Chen *et al.*, 1978; Klein *et al.*, 1978).

Most animal tissues are able to synthesize cholesterol *de novo* from acetate (Dietschy and Wilson, 1970), and there is an extremely rare cholesterol auxotrophy in cultured mammalian cells. Therefore, it is of considerable interest to determine whether rodent myeloma cells require exogenous cholesterol for growth *in vitro*. Sato *et al.* (1984, 1987) have found that P3-X63-Ag8, X63-Ag8.653 (653) and NS-1-Ag4-1 (NS-1) mouse myeloma cells required cholesterol for survival and growth in serum-free medium.

In this paper, we report that IR983F rat myeloma cells and P3X63-Ag8-U1 mouse myeloma cells, the commonly used tumour cells in hybridoma technique, which had been cultivated in serum-free medium containing cholesterol for more than 6 months, still have a cholesterol requirement that can be replaced by human low density lipoprotein but not by mevalonic acid lactone. In addition, we show

Published and © 1991 by The Faculty Press
88 Regent Street, Cambridge, Great Britain

that 32B5 hybridoma cells which had been cultivated in the serum-free medium for more than 6 months do not require exogenous cholesterol for proliferation.

Materials and methods

Maintenance of cells

IR983F (983) rat myeloma cells obtained from Professor H. Bazin, University of Louvain, Belgium (Bazin, 1982), P3X63-Ag8-U1 (P3U1) mouse myeloma cells and 32B5 hybridoma cells secreting monoclonal antibody against asexual blood stages of *Plasmodium vivax* (Li *et al.*, unpublished results) were initially maintained in a humid atmosphere of 7.5% CO_2 at 37°C in basal medium (BM) supplemented with 15% (v/v) newborn calf serum (NCF) (RPS_{15}) or serum-free medium containing cholesterol (DMI). The medium was changed for fresh medium every 3 or 4 days.

Preparation of basal medium and serum-free media

The BM comprised a 1:1 mixture (v/v) of RPMI 1640 (Gibco) and DME/F-12 medium (Sigma) to which was added 2 mM-L-glutamine, 15 mM-HEPES, 2.2 g sodium bicarbonate per litre and 2.0 g D-glucose. DMI was composed of BM and several supplementary factors such as crystalline bovine insulin, human transferrin, sodium selenite, 2-aminoethanol, 2-mercaptoethanol, sodium pyruvate, cholesterol, and BSA-linoleic acid complex (Li *et al.*, 1990). Four serum-free media consisted of the following: (1) DMI without cholesterol (DMI − cholesterol); (2) DMI supplemented with 10 μg/ml human low density lipoprotein (LDL, Sigma) without cholesterol (DMI − cholesterol + LDL); (3) DMI supplemented with 10 μg/ml LDL (DMI + LDL); and (4) DMI alone. Stock solutions of cholesterol (10 mg/ml in 100% ethanol), BSA (50 mg/ml in BM, pH 7.4) and LDL (5 mg/ml) were stored at 4°C. The preparation of BSA-linoleic acid complex was performed as previously described (Li *et al.*, 1990). When added to cell cultures, the stock solutions of BSA, cholesterol and/or LDL were warmed to 40°C, and appropriate volumes of the solutions were individually added, in sequence, to the medium. The final concentration of cholesterol in these serum-free media was 10 μg/ml.

Cell growth assays

The effects of various media on the growth of the rodent parental myeloma cells and hybridoma cells were tested as follows. The cells cultivated in RPS_{15} or in DMI for more than 6 months were harvested and washed in 10 ml Hank's solution three times and subsequently once in BM. They were then seeded in 24-well plates (Costar) at 10^4 cells per well in 1 ml of BM with the appropriate supplements (see Figure legends). The plates were incubated at 37°C in a humid atmosphere of 7.5% CO_2. On the indicated days, cell number and viability determinations in triplicate cultures were made in a standard haemocytometer by counting cells suspended in buffer containing 1% trypan blue.

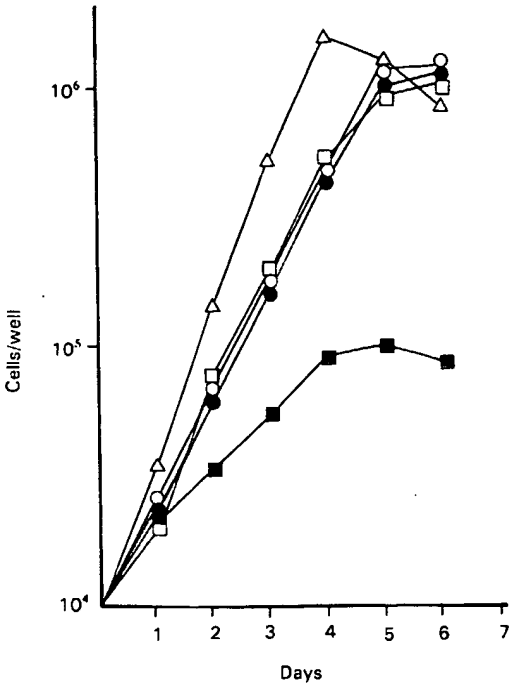


Figure 1 Growth of IR983F cells in serum-free media. IR983F cells maintained in DMI for 6 months were seeded at 1 x 10^4 cells/well in DMI (●), DMI − cholesterol + LDL (○), DMI − cholesterol (■), DMI + LDL (□), or basal medium supplemented with 15% NCS (Δ).

Results

Growth response of 983 rat myeloma cells to several serum-free media

The growth response of 983 cells upon transfer from DMI to several serum-free media is shown in Figure 1. The 983 cells were able to grow and proliferate in various media consisting of either DMI alone, or DMI − cholesterol + LDL, or DMI + LDL or RPS_{15}, but they almost failed to grow in the serum-free medium unsupplemented with cholesterol (DMI − cholesterol) and the morphology of the residual cells was abnormal. After an initial lag period, the 983 cells grew well and reached a maximum on day 6 at the saturation density of 107 x 10^4 in DMI, 112 x 10^4 in DMI − cholesterol + LDL, and 95 x 10^4 in DMI + LDL, respectively. This transition to serum-free media was not accompanied by cell death, which implied that the media were not selecting against sub-populations of 983 cells that required additional serum components for survival and growth.

Growth response of P3U1 mouse myeloma cells to several serum-free media

The growth response of P3U1 cells upon transfer from DMI to several serum-free media is shown in Figure 2. The cells were able to grow and proliferate in DMI, DMI − cholesterol + LDL, DMI + LDL or RPS_{15} media. The P3U1 cells failed to grow and proliferate in DMI − cholesterol. In both DMI and

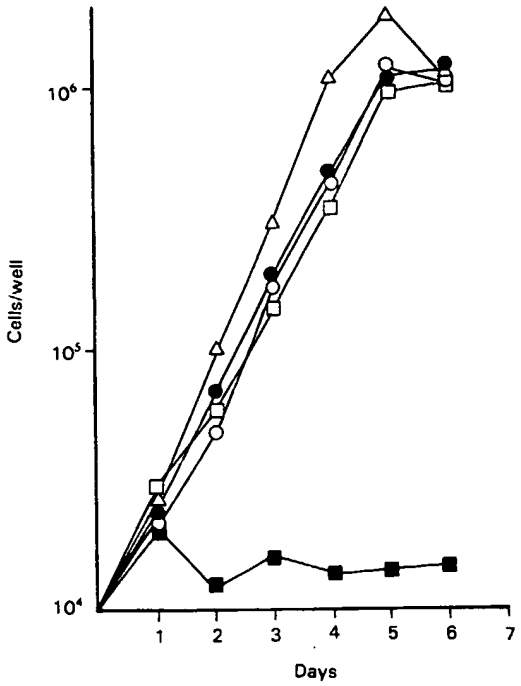


Figure 2 Growth of P3U1 cells in serum-free media. P3U1 cells maintained in DMI for 6 months were seeded at 1 x 10^4 cells/well in DMI (●), DMI − cholesterol + LDL (○), DMI − cholesterol (■), DMI + LDL (□), or basal medium supplemented with 15% NCS (△)

DMI − cholesterol + LDL, P3U1 cells grew well with a doubling time (Td) of 16 h and reached the highest saturation density of 112 x 10^4 on day 6 or 7. These results indicated that cholesterol was essential to them and LDL could also satisfy this requirement.

Growth response of 32B5 hybridoma cells to several serum-free media

Similar experiments were performed to evaluate the growth response of 32B5 hybridoma cells upon transfer from DMI to several serum-free media. The results (Figure 3) showed that the growth response to serum-free culture conditions of 32B5 cells differed from both the rat myeloma cells and the mouse myeloma cells. They grew and proliferated not only in DMI, DMI − cholesterol + LDL, DMI + LDL or RPS_{15} but also in DMI − cholesterol which was comparable to those in DMI and DMI − cholesterol + LDL. Thus, unlike rat or mouse myeloma cells, 32B5 hybridoma cells required no exogenous cholesterol for growth *in vitro*.

Growth responses of the parental myeloma and hybridoma cells to concentrations of cholesterol

Figure 4 shows the growth responses of 983, P3U1 and 32B5 cells to increasing concentrations of cholesterol. The growth curves of the parental myeloma cells exhibited reverse L shapes. Optimal growth of 983 and P3U1 cells occurred in cholesterol concentrations of 15 and 5 μg/ml, respectively. The concentrations of cholesterol had no effect on the growth of 32B5 hybridoma cells.

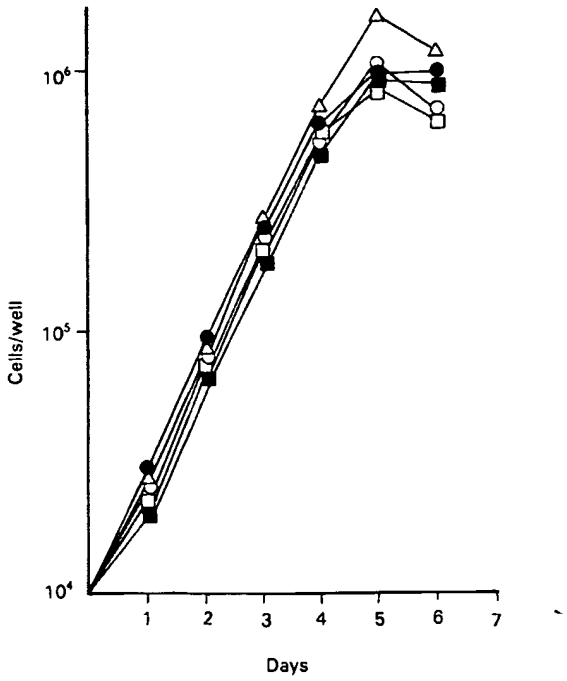


Figure 3 Growth of 32B5 hybridoma cells in serum-free media. 32B5 cells maintained in DMI for 6 months were seeded at 1 x 10^4 cells/well in DMI (●), DMI − cholesterol + LDL (○), DMI − cholesterol (■), DMI + LDL (□), or basal medium supplemented with 15% NCS (△).

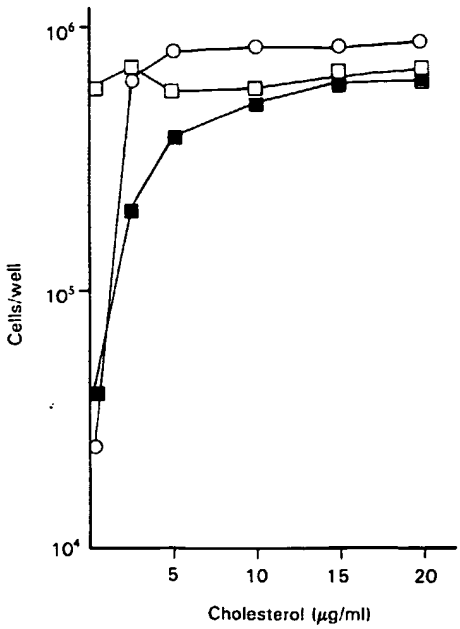


Figure 4 Effect of cholesterol concentration on the growth of IR983F (■), P3U1 (○) and 32B5 (□) cells which were plated at 1 x 10^4 cells/well in DMI − cholesterol supplemented with increasing concentrations of cholesterol.

Effect of mevalonic acid lactone on the growth of 983 and P3U1 cells

In order to determine whether the cholesterol requirement of myeloma cells resulted from deficient 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase, EC1.1.1.23) synthesis or activity, the 983 and P3U1 cells were separately incubated in DMI alone or in DMI − cholesterol supplemented with mevalonic acid lactone. It was found that mavelonic acid lactone in concentrations between 0 and 1.5 mg/ml could not replace cholesterol in stimulating cell growth (data not shown).

Discussion

Most animal cells are capable of synthesizing cholesterol *de novo* from acetate (Dietschy and Wilson, 1970). Only a few mammalian cells express *in vitro* an absolute requirement for exogenous cholesterol. HeLa cells have been reported to require cholesterol for clone growth in the presence of dialysed serum (Sato *et al.*, 1957). Other studies have shown that an established line of porcine kidney cells (Higuchi, 1970) and low passage cultures of human fibroblasts (Holmes *et al.*, 1969) and kidney cells (Gonzalez *et al.*, 1974) required cholesterol for proliferation in serum-free or lipid-deficient medium. Honma *et al.* (1979) found that cholesterol was required by M1 mouse myeloid leukaemia cells for differentiation but not for proliferation in serum-free medium. Esfahani *et al.* (1984) indicated that U937, a human macrophage-like cell line, required cholesterol for growth, and optimal growth occurred in cholesterol concentrations of 10 to 30 μg/ml. Sato *et al.* (1984, 1987) demonstrated that cholesterol was absolutely required for the survival and growth of NS-1, P3-X63-Ag8 and 653 myeloma cells.

In the present study we have found that 983 rat myeloma cells and P3U1 mouse myeloma cells which had been cultivated in serum-free medium containing cholesterol for more than 6 months still required cholesterol *in vitro* for growth and proliferation in serum-free media. Optimal growth occurred in cholesterol concentrations of 15 and 5 μg/ml, respectively. We have confirmed that cholesterol was required for the growth and proliferation of 653 cells and the optimal concentration of cholesterol was 10 μg/ml (data not shown). Moreover, the fact that cholesterol could be replaced by human LDL which is a highly complex molecule composed of apoprotein B, free cholesterol, cholesterol esterified to several fatty acids, and phospholipids (Goldstein and Brown, 1977), was demonstrated. As lipids comprise approximately 75% of the mass of LDL, and cholesterol represents 60% of the total lipids (Goldstein and Brown, 1977), it would be of great interest to test whether other types of phospholipid are able to substitute cholesterol in these experiments. Unfortunately no growth or proliferation of the cells was evident with phosphatidyl choline (date not shown). Since cholesterol levels vary between serum preparations (Olmsted, 1967; Honn *et al.*, 1975), the requirement of these myeloma cells for an exogenous source of cholesterol may be responsible for variations in their growth and viability in serum-supplemented medium.

The basis of the cholesterol requirement of the rodent myeloma cells is unclear. Although the conversion of HMG-CoA to mevalonic acid is a regulated and rate-

limiting step in cholesterol biosynthesis (Brown and Goldstein, 1980; Goldstein and Brown, 1977), a deficiency in HMG-CoA reductase activity may not be responsible because mevalonic acid lactone was not capable of replacing cholesterol in 983 and P3U1 cell cultures. Chen *et al.* (1986) have proved that the NS-1 cells are unable to convert lanosterol to cholesterol. It seems, therefore, that the lesion of cholesterol synthesis in the rodent myeloma cells occurs distal to the formation of mevalonic acid.

Sato *et al.* (1984) reported that SP2/0-Ag14 (SP2/0) cells and the hybridomas derived from NS-1 myeloma cells did not require cholesterol for growth *in vitro* in serum-free medium. Our experimental findings have confirmed that SP2/0 and hybridoma cells derived from P3U1 which had been adapted in DMI for more than 6 months required no exogenous cholesterol for proliferation. However, at the initial stage of transfer from RPS_{15} to DMI, cholesterol obviously influenced the growth of these cells, which was comparable to the effect of human transferrin (Li *et al.*, 1990). At present the biochemical basis of this difference is unknown and currently under investigation.

Acknowledgements

Ji-Liang Li was partially supported in this work by a grant from the National Natural Science Foundation of China (number 3880715) and the Ministry of Public Health of PLA.

References

BAZIN H. 1982. Production of rat monoclonal antibodies with the LOU rats non-secreting IR983F myeloma cell line. *In* Protides of the Biological Fluids. Edited by H. Peeters. pp 615–8. 29th Colloquium. Pergamon Press, Oxford and New York.

BROWN M. S. and Goldstein J. L. 1980. Multivalent feedback regulation of HMG-CoA reductase, a control mechanism co-ordinating isoprenoid synthesis and cell growth. *J. Lipid Res.* **21** 505–17.

BRUCKDORFER K. R., Demel R. R., DeGier J. and Van Deenen L. L. M. 1969. The effect of partial replacements of membrane cholesterol by other steroids on the osmotic fragility and glycerol permeability of erythrocytes. *Biochim. biophys. Acta* **183** 334–45.

CHEN H. W., Heiniger H.-J. and Kandutsch A. A. 1978. Alteration of $^{86}Rb^+$ influx and efflux following depletion of membrane sterol in L-cells. *J. biol. Chem.* **253** 3180–5.

CHEN H. W. and Kandutsch A. A. 1981. Cholesterol requirements for cell growth: endogenous synthesis *versus* exogenous sources. *In* The Growth Requirements of Vertebrate Cells *In Vitro*. pp 327–42. Edited by C. Waymouth, R. Ham and P. Chapple. Cambridge University Press, New York.

CHEN J.-K., Okamoto J. D., Sato J. D., Sato G. H. and McClure D. B. 1986. Biochemical characterization of the cholesterol-dependent growth of the NS-1 mouse myeloma cell line. *Expl Cell Res.* **163** 117–26.

DEKRUYFF B., Demel R. A. and Van Deenen L. L. M. 1972. The effect of cholesterol and epicholesterol incorporation on the permeability and on the phase transition of intact *Acholeplasma laidlawii* cell membranes and derived liposomes. *Biochim. biophys. Acta* **255** 331–47.

DIETSCHY J. M. and Wilson J. D. 1970. Regulation of cholesterol metabolism. *New Engl. J. Med.* **282** 1128–38.

ESFAHANI M., Scerbo L. and Devlin T. M. 1984. A requirement for cholesterol and its structural features for a human macrophage-like cell line. *J. Cell Biochem.* **25** 87–97.

GOLDSTEIN J. L. and Brown M. S. 1977. The low-density lipoprotein pathway and its relation to atherosclerosis. *Ann. Rev. Biochem.* **46** 897–930.

GONZALEZ R., Dempsey M. E., Elliot A. Y. and Fraley E. E. 1974. Studies of sterol synthesis, synthesis regulation and transport in cultured human kidney. *Expl Cell Res.* **87** 152–8.
HEINIGER H.-J., Kandutsch A. A. and Chen H. W. 1976. Depletion of L-cell sterol depresses endocytosis. *Nature* **263** 515–7.
HIGUCHI K. 1970. Requirements for cholesterol, hematin, and lecithin for optimal growth of a porcine kidney cell line. *In Vitro Cell Dev. Biol.* **6** 239.
HOLMES R., Helms J. and Mercer G. 1969. Cholesterol requirement of primary diploid human fibroblasts. *J. cell Biol.* **42** 262–71.
HONMA Y., Kasukabe T., Okabe J. and Hozumi M. 1979. Replacement of serum by insulin, transferrin, albumin, phosphatidyl choline, cholesterol, and some trace elements in cultures of mouse myeloid leukemia cells sensitive to inducers of differentiation. *Expl Cell Res.* **124** 421–8.
HONN K. V., Singley J. A. and Chavin W. 1975. Fetal bovine serum: a multivariate standard. *Proc. Soc. Exp. Biol. Med.* **149** 344–7.
KLEIN I., Moore L. and Pastan I. 1978. Effect of liposomes containing cholesterol on adenylate cyclase activity of cultured mammalian fibroblasts. *Biochim. biophys. Acta* **506** 42–53.
KROES J. and Ostwald R. 1971. Erythrocyte membranes-effect of increased cholesterol content on permeability. *Biochim. Biophys. Acta* **249** 647–50.
KROES J., Ostwald R. and Keith A. 1972. Erythrocyte membranes-compression of lipid phases by increased cholesterol content. *Biochim. biophys. Acta* **274** 71–4.
LANGE Y. and Ramos B. V. 1983. Analysis of the distribution of cholesterol in the intact cell. *J. biol. Chem.* **258** 15130–4.
LI J. L., Ouyang M. H., Chao S., Chang W. S. and Li Y. J. 1990. A serum-free medium adaptive for growth of parental myeloma cells, homohybridomas and heterohybridomas. *Chin. J. Immunol.* **6** 250–3.
OLMSTED C. A. 1967. A physico-chemical study of fetal calf sera used as tissue culture nutrient correlated with biological tests for toxicity. *Expl Cell Res.* **48** 283–99.
POZNANSKY M., Kirkwood D. and Solomon A. K. 1973. Modulation of red cell K^+ transport by membrane lipids. *Biochim. biophys. Acta* **330** 351–5.
ROTTEM S., Cirillo V. P., DeKruyff B., Shinitsky M. and Razin S. 1973a. Cholesterol in mycoplasma membranes. Correlation of enzymic and transport activities with physical state of lipids in membranes of *Mycoplasma mycoids* var. capri adapted to grow with low cholesterol concentrations. *Biochim. biophys. Acta* **323** 509–19.
ROTTEM S., Yashouv J., Ne'eman Z. and Razin S. 1973b. Cholesterol in mycoplasma membranes. Composition, ultrastructure and biological properties of membranes from *Mycoplasma mycoides* var. capri cells adapted to grow with low cholesterol concentrations. *Biochim. biophys. Acta* **323** 495–508.
SATO G. H., Fisher H. W. and Puck T. T. 1957. Molecular growth requirements of single mammalian cells. *Science* **126** 961–4.
SATO J. D., Kawamoto T., McClure D. B. and Sato G. H. 1984. Cholesterol requirement of NS-1 mouse myeloma cells for growth in serum-free medium. *Mol. Biol. Med.* **2** 121–34.
SATO J. D., Kawamoto T. and Okamoto T. 1987. Cholesterol requirement of P3-X63-Ag8 and X63-Ag8.653 mouse myeloma cells for growth *in vitro*. *J. expl Med.* **165** 1761–6.
SODERGREN A., Marshall J. D. and Heiniger H.-J. 1983. Suppression of endocytosis in polyclonally activated Con A-stimulated T lymphocytes by 25-hydroxycholesterol. *Cell Immunol.* **76** 268–75.

Accepted 20 June 1991

Canella, Karen

From: STIC-DOCS
Sent: Wednesday, January 31, 2007 12:39 PM
To: Canella, Karen
Subject: ILL REQUEST (616635) 10727432

Your Request Number: 21757663
for "Enhanced formation of mouse hybridomas without hat" from IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY : JOURNAL OF THE TISSUE CULTURE ASSOCIATION
Relais Request Number: REG-14025529

http://public.nlm.nih.gov/projects/relais/3891908.pdf

This document contains 7 pages (including the cover page), is 1,087KB, was posted on January 31, 2007 at 10:26:37 AM and will be available for 7 days.

Cytotechnology **18**: 207–217, 1996.

The use of serum-free medium for the production of functionally active humanised monoclonal antibody from NS0 mouse myeloma cells engineered using glutamine synthetase as a selectable marker

M. J. Keen and C. Hale
Biological Research Division, Wellcome Research Laboratories, Langley Court, Beckenham, Kent, UK

Received 15 March 1995; accepted 23 August 1995

Key words: serum-free, mouse myeloma, NS0 cells, humanised monoclonal antibody, glutamine synthetase

Abstract

A protein-free growth medium (W38 medium) had previously been developed for the NS0 mouse myeloma cell line which is cholesterol-auxotrophic. This paper describes the development of a protein-free growth medium for NS0 cells expressing humanised monoclonal antibody using GS (glutamine synthetase) as a selectable marker. Several GS-engineered NS0 cell lines expressing humanised monoclonal antibody grew in a modification of W38 medium which maintained GS-selection, supplemented with cholesterol, phosphatidylcholine and β-cyclodextrin. Further studies showed that additional glutamic acid, asparagine, ribonucleosides and choline chloride improved cell growth. Amino acid analysis identified a number of amino acids that were being depleted from the culture medium. NS0 cell lines 9D4 and 2H5 expressing CAMPATH-1H* were adapted to enable them to grow serum-free in the absence of cholesterol and β-cyclodextrin. Cholesterol-independent 9D4 (9D4.CF) cells grown in shake flask culture using an enriched protein-free medium (WNSD medium), supplemented with human recombinant insulin (Nucellin), reached a maximum cell density to 1.86×10^6 cells ml^{-1} producing 76.6 mg l^{-1} of antibody. CAMPATH-1H antibody produced using serum-free medium was found to be functionally active *in vitro* in the Antibody Dependant Cellular Cytotoxicity (ADCC) assay.

Abbreviations: C – cholesterol; CD – cyclodextrin; dhfr – dihydrofolate reductase; F68 – Pluronic F68; GS – glutamine synthetase; MSX – methionine sulphoximine; P – phosphatidylcholine; PC-FBS – phosphatidylcholine, cholesterol and foetal bovine serum; RPMI – RPMI 1640 medium, ADCC – Antibody-dependant cellular cytotoxicity

Introduction

NS0 (Galfre and Milstein, 1982) is a cholesterol-auxotrophic (Keen and Steward, 1995) mouse myeloma cell line that does not contain endogenous glutamine synthetase (GS) (Brown *et al.*, 1992) and is therefore unable to synthesise glutamine from other amino acids. For this reason NS0 cells have an essential requirement for exogenous glutamine and are particularly well suited for use in recombinant gene technology which utilises glutamine synthetase as a selectable marker (Bebbington *et al.*, 1992). The GS expression system exploits the lack of cellular GS in the host cell line using a plasmid containing both the GS gene and the product gene. The plasmid contains the DNA encoding for the antibody heavy and light chains driven by the strong hCMV promoter regions and DNA encoding for glutamine synthetase controlled by the weak SV40 promoter. Transfected cells can be isolated by culturing in a selection medium which lacks glutamine. The GS and product gene can be co-amplified

* CAMPATH is the registered trade mark of Wellcome Group Companies.

by exposure with methionine-sulphoximine (MSX), a GS-inhibitor.

Monoclonal antibodies from different mammalian species can be humanised by grafting the complementary determining regions (CDRs) from the antibody of one species on to a suitable human antibody donor framework (Winter and Milstein, 1991). Humanised antibodies are expected to be of greater therapeutic use in man than chimeric or murine antibodies which are known to generate an anti-species or an anti-frame work immune response, such as the human anti-mouse antibody response (HAMA response) (Wallace *et al.*, 1994; Isaacs, 1990). An additional advantage of humanisation is that the isotype of the human Fc framework can be selected to support the desired effector functions.

Cloned GS-engineered NS0 cell lines expressing CAMPATH-1H or humanised anti-CD2 monoclonal antibody were used in this study. CAMPATH-1H is a humanised IgG1 monoclonal antibody (Riechmann *et al.*, 1988) which binds to the CD52 antigen, which is an abundant glycoprotein present on the vast majority of human lymphocytes (Hale *et al.*, 1990), monocytes and most lymphoid malignancies (Hale *et al.*, 1988b). CAMPATH-1 antibodies have been used successfully in the therapy of transplant rejection (Hale *et al.*, 1988a), rheumatoid arthritis (Isaacs *et al.*, 1992) and non-Hodgkins lymphoma (Hale *et al.*, 1988b). Previously, CAMPATH-1H had been engineered into the CHO DUK B11 cell line (Urlaub and Chasin, 1980) using a dihydrofolate reductase (dhfr) selection system (Page and Sydenham, 1991). CAMPATH-1H is known to initiate cell death through antibody-dependant cellular cytotoxicity (ADCC) (Hale *et al.*, 1985; Dyer *et al.*, 1989).

Fully defined protein-free media offers a number of potential advantages for manufacture of antibodies, vaccines and other therapeutic products from mammalian cells relating to cost, reproducibility, regulatory considerations and purification of product. Protein-free culture media are now available that will support the growth of some hybridoma and myeloma cell lines. Cells that require exogenous cholesterol, such as NS0, will not grow in these media. We have described the growth of NS0 cells in W38 protein-free medium containing cholesterol rich lipid with β-cyclodextrin and their subsequent adaptation to cholesterol-independence (Keen and Steward, 1995). In this study a protein-free medium was developed for GS-engineered NS0 cells which supported cell growth and the production of functionally active antibody.

Materials and methods

Media and reagents

The following materials were used: Glutamine, non-essential amino acids, and trypan blue were obtained from ICN-Flow (High Wycombe, UK); RPMI 1640 product 31870–025 (RPMI), Dulbecco's medium product 21969–035 (DMEM), DMEM without ferric nitrate product 73-860–54 and Iscove's modified Dulbecco's medium (Iscove's) product 21980–032 from Life Technologies (Paisley, UK); dialysed and non-dialysed New Zealand foetal bovine serum (FBS) from Bioclear (UK); ferric citrate product 28381 from BDH (Poole, UK); tissue culture plastic from Costar (Badhoevedorp, Holland) and Erlenmeyer flasks from Corning (New York, USA). The cyclodextrins used were a generous gift from Wacker Chemicals (Munich, Germany). All other Chemicals and supplements were obtained from Sigma (Poole, UK).

Ferric citrate was dissolved at 10 mg ml^{-1} in boiling water, allowed to cool, then 0.2 μm filtered using a Millex GV filter (Millipore, France). Cyclodextrins were prepared as a 10 mg ml^{-1} solution in water then 0.2 μm filtered. WNSA, WNSB and WNSD media (Table 2) were prepared by the Medium Production Unit, Wellcome (method of preparation available on request).

Preparation of vesicles containing phosphatidylcholine and cholesterol

Phosphatidylcholine (P) and cholesterol (C) were dissolved at 10 mg ml^{-1} in absolute ethanol. A cholesterol rich lipid solution (CR-lipids) was prepared by mixing P and C in a ratio of 2:1 with 0.25 mg ml^{-1} α-tocopherol to give a final total concentration of 10.25 mg ml^{-1}. For experimental purposes this solution was treated as being sterile. Both cholesterol and phosphatidylcholine have a very low solubility in aqueous solution; cholesterol forms a precipitate while phosphatidylcholine becomes uniformly suspended in solution. When phosphatidylcholine and cholesterol are mixed together in ethanol, then rapidly added to aqueous solution small unilamellar vesicles are formed (Stryer, 1988). Vesicles were thus prepared in the culture medium by the addition of CR-lipids to give a final concentration of 10.25 mg l^{-1}.

GS-engineered cell lines and routine culture conditions

The serum-containing GS-selection medium used ('select' medium) was a modification of Celltech 'GS-selection medium' (Bebbington and Yarrington, 1989) which contained DMEM without ferric nitrate with 60 mg l^{-1} glutamic acid (G) and asparagine (A), 7 mg l^{-1} adenosine, guanosine, cytidine and uridine, 2 mg l^{-1} thymidine, ICN-Flow non-essential amino acids at single strength and 5% dialysed FBS. Only cells which express GS are able grow in this medum.

In order to express antibody in NS0 cells, the antibody heavy and light chain cDNA's were cloned into glutamine synthetase selection based vectors obtained from Celltech Ltd, Slough, UK. This construct was transfected into the NS0 cell line by electroporating twice using a Bio Rad Gene Pulser set at 1500 volts and 3 μF. The transfected cells were cultured in using GS 'select' medium. Screening by ELISA assay identified the colonies that produced the highest level of antibody. High secreting single colonies were expanded, amplified by exposure to various concentration (5–50 μm) of MSX, then dilution cloned.

NS0 9D4.5A11 (9D4) and 9D4.4F8 (4F8) expressing CAMPATH 1H were derived from the same primary well and amplified with 5 μM MSX. NS0 2H5 (2H5) expressing CAMPATH 1H was amplified with 10 μM MSX. Specific antibody production rates (SPR) for 9D4, 4F8 and 2H5 were 11, 17 and 4 μg 10^6 $cells^{-1}$ day^{-1}. The SPR was determined over 24 hours, in a sub-confluent static culture using serum-containing 'select' medium. The 8C9.50B5 (8C9) cell line which expressed humanised anti-CD2 antibody was not amplified with MSX. These cells were routinely grown in static culture using 75 cm^2 flasks containing 30 ml of serum-containing 'select' medium with MSX which was incubated at 37 °C with 7.5% CO_2. The cells grew slowly, lightly attached to the plastic reaching a maximal concentration of around 6×10^5 cells ml^{-1}. Every few days the cells were diluted to approximately 10^5 cells ml^{-1} in fresh growth medium . The adherent cells were easily removed from the plastic by firmly tapping the side of the flask. Non-engineered NS0 cells were grown in static culture using DMEM, 5% FBS and 2 mM l-glutamine (NS0 growth medium).

Growth curves were performed using duplicate shake flask cultures. 250 ml Erlenmeyer flasks containing 35 ml of culture were incubated at 37 °C in an Innova shaking incubator (New Brunswick Scientific, USA) and shaken at 100 revolutions per minute (rpm). The cultures were re-gassed daily for half a minute with 5% CO_2 and a sample removed for cell counting and antibody assay. Daily gassing ensured that cell growth was not inhibited by O_2 depletion or CO_2 build up.

Washing procedure and cell counting method

Cells grown in serum-containing medium were washed by pelleting at 1200 × g for 5 minutes at +4 °C, resuspended in protein-free medium without addition, pelleted again, resuspended in fresh medium at 2×10^6 cells ml^{-1}, then diluted to the required concentration in the media under test. All media were pre-warmed to room temperature before use.

Culture samples were diluted with an equal volume of 0.1% w/v isotonic trypan blue (trypan blue) , mixed by pipetting, then counted using trypan blue exclusion. In serum-containing static cultures the cells were first dislodged by tapping the side of the flask.

Amino acid analysis

Amino acid composition of the culture medium was analysed before and after cell growth in order to determine usage by the cells. Centrifuged medium samples were first hydrolysed by addition of HCl to a final concentration of 0.1 M and proteins removed by precipitation with 80% ethanol. The supernatant was dried (speedvac concentrator), reconstituted with a 250 ppm tripotassium EDTA solution and passed through a filter with a 10,000 molecular weight cut off. The amino acid composition was then determined using an Applied Biosystems model 420A dedicated derivitiser/amino acid analyser.

Detection of antibody levels by ELISA and functional activity by ADCC

Culture supernatant was clarified by microfuge centrifugation at 13 000 rpm and stored at +4 °C for up to 2 weeks prior to assay. The amount of humanised antibody was determined using a sandwich ELISA assay. The wells of a 96 well maxisorp ELISA plate (Nunc, Roskilde, Denmark) were coated with 2 μg ml^{-1} sheep anti-human IgG heavy and light chain (Seralab, UK, cat. no. SDL2015) in phosphate buffered saline (PBS). The excess protein binding sites were blocked with diluent (1% Bovine serum albumin fraction V in PBS). The binding of triplicate antibody samples to the coated

wells was detected using peroxidase conjugated sheep anti-human IgG and then measured by the colour generated from the tetra-methyl benzidine substrate (Sigma, Poole, UK). The reaction was stopped with 2 M H_2SO_4 and the colour measured at an absorbance of 450 nm. The amount of antibody in the test samples was quantified from a curve generated using a purified CAMPATH-1H antibody standard. Between each step of the ELISA the plates were washed 6 times with wash buffer (PBS with 0.05% Tween 20 detergent).

For the Antibody dependant cellular cytotoxicity assay (ADCC) peripheral blood mononuclear cells (PBMC) were separated from defibrinated fresh human blood by centrifugation over a lymphoprep (Nycomed) gradient. Wien 133, a human B cell lymphoma cell line (Nacheva *et al.*, 1987) expressing the CD52 antigen on its cell surface membrane was grown in log phase, labelled with ^{51}Cr, washed, resuspended at 2×10^5 ml^{-1}, then aliquoted into 96 well 'U' bottomed tissue culture plates at 50 μl well^{-1}. Wien 133 were cultured using Iscove's medium, 10% FBS and 2 mM l-glutamine (Wien growth medium). CAMPATH-1H antibody dilutions (50 μl) in Wien growth medium, were added to triplicate wells in the plate. Nine control wells without antibody were also included in each assay. The plates were incubated at 37 °C for 1.5 hours in a humid CO_2 incubator prior to the addition of 100 μl of PBMC effector cells at a concentration of 2.5×10^6 ml^{-1}, in Wien growth medium, to each antibody-containing well. This resulted in a ratio of 25 effector cells for each target cell. The effector cells were also added to one triplicate set of control wells (non-antibody mediated lysis control), Wien growth medium alone was added to yet another set of control triplicates (spontaneous lysis control) and 100 μl of 1% w/v Triton X100 detergent in H_2O was added to the final set of control wells (total release control). The plates were spun for 5 minutes at 1500 rpm before being replaced at 37 °C for 5 hours. At the end of the incubation period 100 μl of medium was removed from each well and counted in a γ counter (Wallac Wizard, model 1470, Turku, Finland). The level of specific release was calculated as follows:

$$\% \text{ specific release} = \frac{(\text{mean cpm. antibody mediated release}) - (\text{mean cpm. spontaneous release}) \times 100}{(\text{mean cpm. total release}) - (\text{mean cpm. spontaneous release})}$$

Results

Growth characteristics in serum-containing 'select' medium

Each of the GS-engineered NS0 cell lines grew differently in serum-containing 'select' medium. Duplicate static cultures of line 9D4 seeded at 10^5 cell ml^{-1} reached an average density of 5.75×10^5 cells ml^{-1}, while line 8C9 reached an average density of 12.9×10^5 cells ml^{-1}. NS0 9D4 cells were pre-adapted to suspension growth in 'select' medium. Duplicate shake flask cultures of these cells, seeded at 10^5 cells ml^{-1}, reached an average density of 6.75×10^5 cells ml^{-1} after 4 days with an average antibody level 12.01 mg l^{-1} after 6 days.

The protein-free growth of GS engineered NS0 cells

GS-engineered cells require glutamine-free culture medium in order to maintain selection pressure. Glutamine synthetase produces glutamine directly from glutamic acid and indirectly from other amino acids such as asparagine. The catabolism of glutamine is essential for the *de novo* synthesis of ribonucleosides. GS-selection media should therefore contain glutamic acid, asparagine and ribonucleosides but be free of glutamine. W38 protein-free medium (Keen, 1995) supplemented with CR-lipids and β-cyclodextrin supports high density growth of non-engineered NS0 cells. A modification of W38 medium, designated WNSA, was produced (Table 1), which was free of glutamine and contained additional glutamic acid, asparagine, adenosine, guanosine, cytidine, uridine and thymidine.

9D4 and 2H5 cells were adapted to protein-free growth in 25 cm^2 flasks. Cells grown in serum-containing 'select' medium were washed using WNSA medium, resuspended at 10^5 cells ml^{-1} in 5 ml WNSA medium supplemented with CR-lipids and cyclodextrin (WNSA + lipids), then incubated at 37 °C with 7.5% CO_2. Every few days the cells were subcultured by dilution with fresh medium taking great care to ensure that the cells did not overgrow. Both 9D4 and 2H5 readily adapted to protein-free growth (Tables 2A and 2B), with the non-viability remaining between 10% to 30% throughout. The high non-viability may have been due to cell death occurring immediately after

Table 1. Composition of WNSA and WNSD select medium. The final osmolality of these media was between 330 and 350 mOSM. The differences in WNSD medium are highlighted

	Final concentration of medium components mg l^{-1}				
	WNSA	WNSD		WNSA	WNSD
Amino acids			**OTHER COMPONENTS**		
L-Alanine	0	0			
L-Arginine	142	**192**	EDTA[a]	6	6
L-Asparginine	85	**505**	Oxalacetic acid	2	2
L-Aspartic acid	10	**20**	Progesterone	0.006	0.006
L-Cystine	61.7	61.7	Sodium nitroprusside	5	5
L-Glutamic acid	70	**490**	Taurine	30	30
Glycine	20	**30**	Tween-80	0.2	0.2
L-Histidine	28.5	**58.5**	Glucose	6250	**3000**
L-Hydroxy-proline	10	10	Hypoxanthine	0.272	0.272
L-Isoleucine	77.4	**177.4**	Adenosine	7	**14**
L-Leucine	77.4	**177.4**	Guanosine	7	**14**
L-Lysine	93	**193**	Cytidine	7	**14**
L-Methionine	22.5	**82.5**	Uridine	7	**14**
L-Phenylalanine	40.5	**60.5**	Phenol red	10	10
L-Proline	10	10	Putrescine	2.2	2.2
L-Serine	36	36	Pyruvic acid	165	165
L-Threonine	57.8	57.8	Thymidine	2.41	**4.81**
L-Tryptophan	10.5	**30.5**	MOPS buffer	3000	3000
L-Tyrosine	57.2	57.2	Sodium bicarbonate	2850	2850
L-Valine	56.8	**116.8**			
			TRACE ELEMENTS		
VITAMINS			Aluminium chloride	0.001	0.001
δ-Pantothenic acid	2.1	2.1	Barium chloride	0.002	0.002
Choline chloride	4.2	**104.2**	Calcium chloride	167	167
Flavin adenine-dinucleotide	0.02	0.02	Chromium potassium-sulphate	0.001	0.001
Folic acid	2.5	2.5	Copper sulphate	0.0025	0.0025
Glutathione	0.5	0.5	Ferric citrate	1	1
i-Inositol	21	21	Ferrous sulphate	0.8	0.8
Nicotinamide	2.5	2.5	Lithium chloride	10	10
P Aminobenzoic acid	0.5	0.5	Magnesium sulphate	150	150
Pyridoxin	2.5	2.5	Molybdic acid	0.0001	0.0001
Riboflavin	0.3	0.5	Nickel chloride	0.0002	0.0002
Thiamin	2.5	2.5	Potassium bromide	0.0001	0.0001
α-tocopherol	0.00025	0.00025	Potassium chloride	400	400
Cyanocobalamin	0.68	0.68	Potassium iodide	0.00001	0.00001
Biotin	0.1	0.1	Rubidium chloride	0.00001	0.00001
			Silver chloride	0.000005	0.000005
OTHER COMPONENTS			Sodium chloride	6200	6200
Adenine	0.4	0.4	Sodium fluoride	0.004	0.004
Citric acid	25	25	Sodium phosphate	471	471
Phosphatidylcholine	1	1	Sodium selenite	0.03	0.03
Ethanolamine	3	3	Stannous chloride	0.0001	0.0001
			Zinc sulphate	0.8	0.8

[a] EDTA = Ethylenediaminetetraacetic acid-disodium salt.

Table 2. Adaptation to serum-free growth

Pass number	day	viable	non-viable	subcultured	% residual FBS
		Cell count × 10^{-5} cells^{-1}			
Table 2A, line 9D4					
1	7	5.3	4.6	1 to 8	0
2	13	2.0	0.65	1 to 4	0
3	18	2.45	0.6	1 to 3	0
4	20	2.4	0.25	1 to 3	0
5	25	3.0	0.65	1 to 5	0
6	30	3.0	0.8	cryopreserved	
Table 2B, line 2H5					
1	7	7.15	0.5	1 to 8	0
2	13	2.3	0.7	1 to 4	0
3	18	6	0.95	1 to 3	0
4	20	3.8	0.65	1 to 3	0
5	25	4.4	0.95	1 to 5	0
6	30	3.5	0.9	cryopreserved	
Table 2C, line 4F8					
1	5	5.0	2.95	1 to 5	0.2
2	10	9.1	2.7	1 to 5	0.04
3	14	4.2	1.1	1 to 5	0.008
4	19	3.7	1.3	1 to 5	0.016
5	24	10.9	3.2	1 to 5	0 (cells washed)
6	28	5.4	0.7	cryopreserved	

Tables 2A and 2B. The NS0 **9D4** and **2H5.10** cell line secreting CAMPATH-1H antibody **were washed to remove serum** then adapted to growth in **WNSA** protein-free medium containing Nucellin, β-cyclodextrin and cholesterol-containing lipids.
Table 2C. The NS0 **4F8** cell line secreting CAMPATH-1H antibody was adapted to growth in **WNSB** protein-free medium containing recombinant insulin, β-cyclodextrin and cholesterol-containing lipids. The **residual FBS was diluted on sequential passage** then finally remove by washing the cells after 24 days in culture.

subculture and to the relatively slow growth rate.

An attempt was made to improve adaptation by using a weaning process. Cells in serum-containing medium were diluted in WNSA + lipids without washing and the residual serum sequentially diluted out as the cells were passaged. This method of adaptation (Table 2C) was also successful but seemed to offer no benefit over direct transfer to protein-free growth. Out of 11 different GS-engineered cell lines that were transferred to grow in WNSA + lipids, 10 adapted to protein-free growth. The other line grew only in WNSA + lipids containing glutamine suggesting that it was unable to synthesise glutamine when cultured in this medium. 9D4 , 2H5 and 8C9 were sequentially passaged in WNSA + lipids. The addition of 5 mg l^{-1} of human recombinant insulin (Nucellin) slightly increased the cell growth rate and improved viability (data not shown). For this reason Nucellin was then routinely added to the medium.

Enrichment of WNSA protein-free medium with additional glutamic acid, asparagine and ribonucleosides (select additives) improves growth and antibody secretion

WNSA medium was enriched to try and improve cell growth. Figure 1 shows the growth of serum-free adapted 9D4, 2H5 and 8C9 in 5 ml static cultures with WNSA medium containing additional select additives. In the cultures with 'extra ribonucleosides' or 'extra

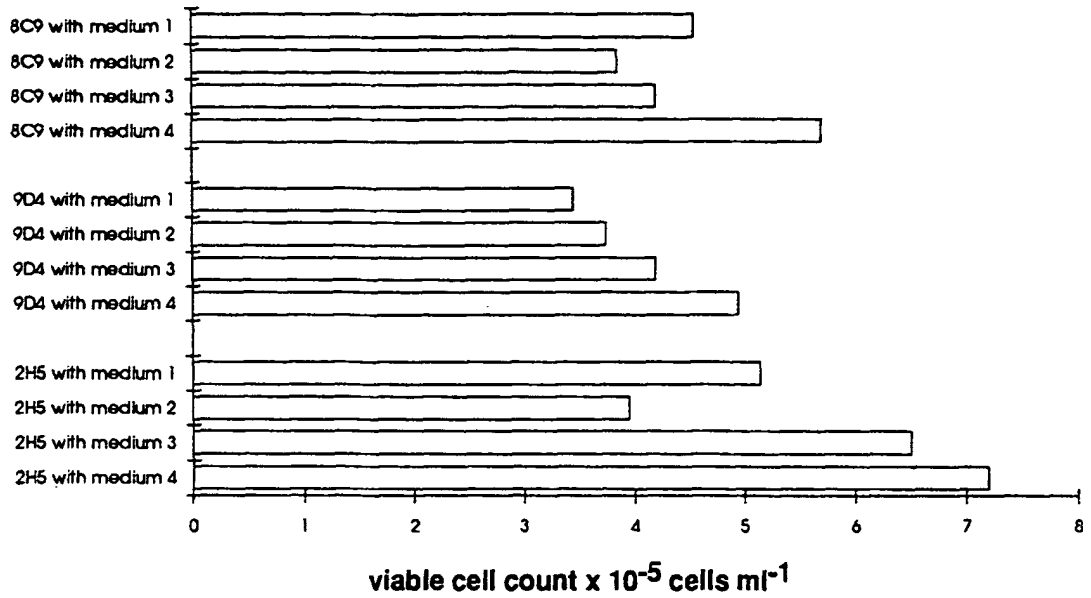


Fig. 1. Effect of adding extra amino acids and ribonucleosides on the growth of three GS-engineered NS0 cell lines in medium 1. WNSA protein-free medium with lipid, β-cyclodextrin and recombinant insulin (WNSA + lipids), medium 2. WNSA + lipids with extra ribonucleosides, medium 3. WNSA + lipids with extra G+A and medium 4. WNSA + lipids with extra G+A and ribonucleosides. Mean counts of duplicate shake flask cultures. G + A = glutamic acid and asparagine.

G+A' the level of ribonucleosides was doubled, glutamic acid was at 190 mg l^{-1} and asparagine at 205 mg l^{-1}. Duplicate 5 ml cultures seeded at 0.5×10^5 cells ml^{-1} in 25 cm^2 flasks were incubated with 7.5% CO_2. For all three cell lines, best growth occurred in the presence of additional G+A and additional ribonucleoside ('fully supplemented' medium). The cell concentration in the 'fully supplemented' 9D4, 2H5 and 8C9 cultures were respectively 4.95, 7.2 and 5.7×10^{-5} cells ml^{-1}, with antibody concentrations of 29.1, 13.8 and 8 mg l^{-1}.

Amino acid and glucose utilisation of GS-engineered NS0 in WNSA medium

In order to examine nutrient depletion, media samples pooled from duplicate cultures of 9D4, 2H5 or 8C9, in 'fully supplemented' WNSA medium were harvested after 6 days growth and the amino acid, glucose and lactate composition analysed (Table 3). The nutrient levels detected after 6 days growth were compared with the levels detected in unused culture medium. Several amino acids notably glutamic acid, asparagine, histidine, valine, methionine, cystine, isoleucine and leucine were heavily depleted with both glutamic acid and leucine being practically exhausted. In contrast glucose levels in the medium did not appear to be growth limiting.

Adaptation of NS0 9D4 to cholesterol independence

One aim of this study was to achieve cell growth in a medium free from animal derived components. Cholesterol is the only animal derived component in WNSD medium. However, NS0 cells have an essential requirement for cholesterol, which is extracted from Sheep wool. The use of β-cyclodextrin and lipid vesicles to supply the cholesterol requirement of NS0 cells caused several problems. Addition of cholesterol-containing vesicles to the medium resulted in lipid precipitation and increased cell death (data not shown). The shelf life of media containing vesicles was limited as after a few days +4 °C storage a lipid precipitate slowly formed. A precipitate could also be seen in slow growing cultures, after several days incubation, resulting in reduced cell viability. The precipitation problem could be reduced by the use of cholesterol:methyl-β-cyclodextrin complex (Sigma product C3175).

It was decided to adapt the 2H5 and 9D4 cell lines to cholesterol-independence using a method similar to that used for non-engineered NS0 cells (Keen and Steward 1995). Briefly, 0.1 ml of CR-lipids at a total

Table 3. Amino acid (aa) utilisation by the GS-engineered NS0 cell lines

	Actual amino acid of content of unused medium	Amino acid levels detected by the analyser in			
		unused medium	8G9 supn't	2H5.10 supn't	9D4 supn't
L-aspartic acid	10	26.3	11.3	10.3	13.3
L-glutamic acid	190	170.7	**0**	**0**	**0**
L-asparagine	205	205.8	**35.6**	**4.6**	136
L-serine	36	44.6	24.7	41	33.9
L-glutamine	0	6.9	4.4	8.8	9.9
L-glycine	20	21	15.4	15	16.1
L-histidine	28.5	21.3	**8.1**	**12**	**9.3**
L-taurine	30	23.4	21.9	23.4	22.5
L-arginine	142	121.4	78.7	90.9	49.2
L-threonine	57.8	61.3	35.7	44.6	35.1
L-alanine	0	3.1	37.8	81.7	44.1
L-proline	10	9.2	5.8	21	4
L-tyrosine	57.2	44.3	24.9	29	28.5
L-valine	56.8	54.7	**9.7**	**14**	**12.9**
L-methionine	22.5	24.2	**5.6**	**6**	**6.7**
L-cystine, L-cysteine	61.7	39.0	**15.0**	**14.4**	**14.4**
L-isoleucine	77.4	76	**9.2**	**10.5**	**5.2**
L-leucine	77.4	74.3	**1.6**	**4.3**	**0**
L-phenylalanine	40.5	49.1	24.3	21.9	17.7
ornithine	0	0	6.8	9.7	40.1
L-tryptophan	10.5	3.6	4.6	4.1	2.6
L-lysine	93	79.9	32.1	40.5	35.8
glucose	6250	6164	ND	ND	4490
lactate	0	0	ND	ND	1350

The first column shows the actual level of each amino acid in unused culture medium. The other four columns show the levels detected in unused medium and in culture medium harvested from cultures of NS0 8G9, 2H5.10 and 9D4 cells grown in static culture for 6 days using 'supplemented' WNSA medium. The highlighted amino acid values are much lower than in the unused culture medium, indicating that they were being depleted from the medium.

lipid concentration of 10.25 mg ml^{-1} in ethanol was added to 10 ml of dialysed FBS (CR-FBS). Cells growing in WNSA + lipid medium were subcultured into WNSA medium containing 1% CR-FBS, but lacking CR-lipids or β-cyclodextrin. The 9D4 cells were subsequently subcultured four times with 0.3% CR.FBS, then cultured serum-free and cholesterol-free, whereas the 2H5 cells were sequentially subcultured once with 1% CR.FBS, twice with 0.5%, twice in 0.15%, then grown cholesterol-free. The adapted cell lines were renamed 9D4.CF and 2H5.CF respectively.

Enriched WNSA medium with additional amino acid, ribonucleosides and choline gave increased biomass and antibody secretion

The effect of further medium enrichment on cell growth was investigated using potentially beneficial additives identified in the nutrient depletion studies. A modification of WNSA protein-free medium (WNSB) was prepared in which the glucose level was reduced to 3 g l^{-1}, asparagine increased to 265 mg l^{-1} and glutamic acid increased to 250 mg^{-1}. A 100× concentrate amino acid supplement (GS-aa) was prepared in water containing 10 mg ml^{-1} isoleucine, leucine and lysine, 6 mg ml^{-1} valine and methionine, 5 mg ml^{-1} arginine, 3 mg ml^{-1} histidine, 2 mg ml^{-1} phenylalanine and tryptophan with 1 mg ml^{-1} glycine and

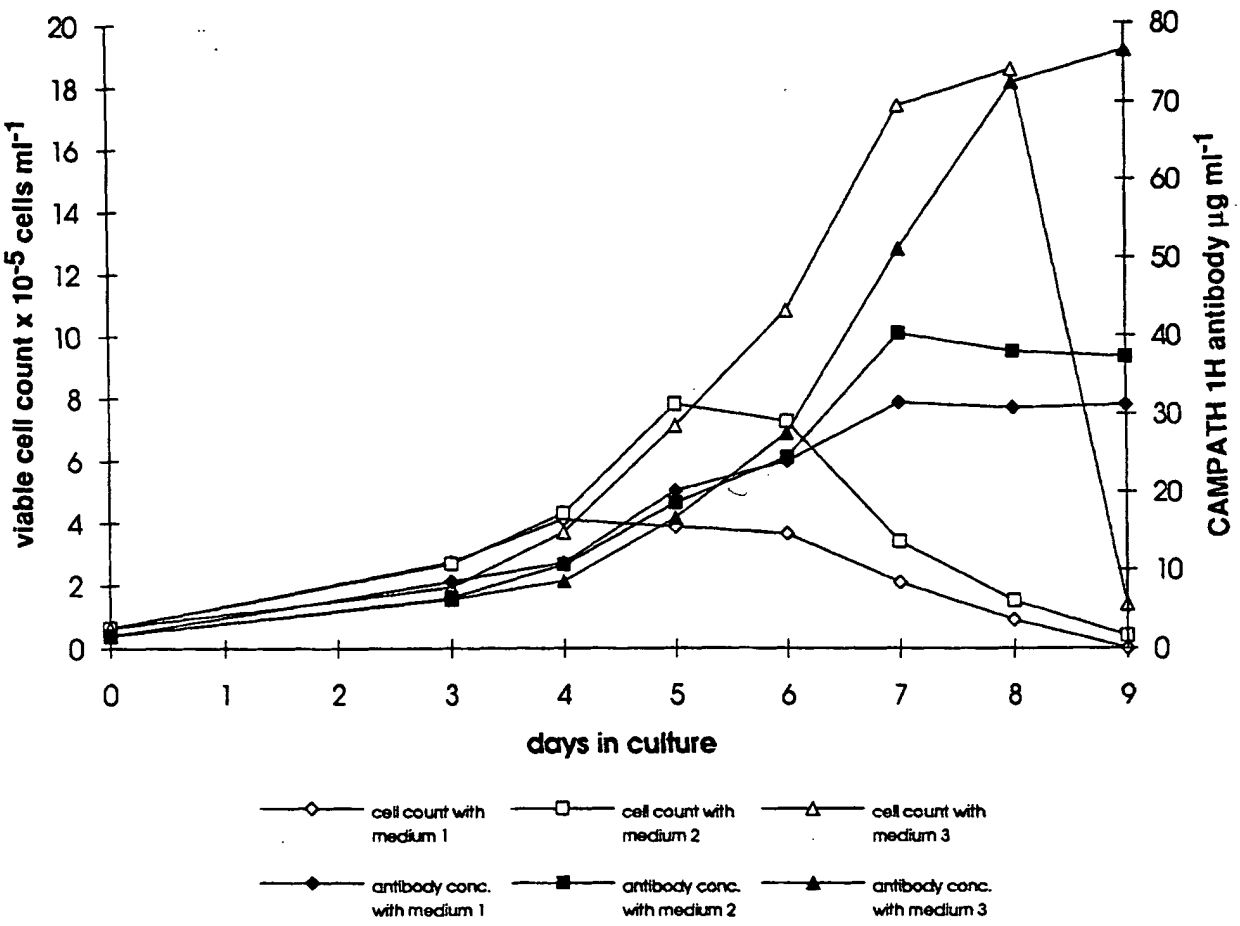


Fig. 2. NS0 9D4.CF cell growth and antibody production in medium 1. WNSB medium with Nucellin, medium 2. WNSB medium with Nucellin, GS-aa, extra ribonucleosides and extra G + A or medium 3. WNSB medium with Nucellin, GS-aa, extra ribonucleosides, extra G + A and choline chloride. Mean of duplicate shake flask cultures. G + A = glutamic acid and asparagine.

aspartic acid. In previous unpublished studies it was found that choline improved Y0 mouse myeloma and Chinese hamster ovary cell growth. A 100× concentrate solution containing 100 mg ml^{-1} choline chloride was prepared in water. These solutions were sterilised by filtration.

Figure 2 compares the growth of 9D4.CF cells in WNSB medium with Nucellin supplemented with additional nutrients using duplicate shake flasks seeded with 10^5 cells ml^{-1}. Medium 2 and 3 contained double ribonucleosides, 490 mg l^{-1} glutamic acid and 505 mg l^{-1} asparagine. The choline chloride in medium 3 had a dramatic effect on growth increasing the maximal cell density to 18.55 × 10^5 cells ml^{-1} with 76.6 mg l^{-1} antibody. The composition of medium 3 (without Nucellin) was renamed WNSD (Table 1). In WNSD medium with added Nucellin the cell density and accumulated antibody was far higher than in serum-containing select medium. Amino acid analysis of supernatant from the 7 days culture with WNSD with Nucellin (Figure 3) showed that there was a high rate of depletion of asparagine, glutamic acid, isoleucine and leucine. From this data it seemed probable that a lack of glutamic acid, isoleucine and leucine was inhibiting cell growth. Unfortunately, increasing the amino acid levels resulted in toxicity and inhibited cell growth. Removal of Nucellin had no detectable effect on cell growth or antibody production (data not shown). Electronmicrographs of cells in late log phase showed a condensation of chromatin that is characteristic of death by apoptosis. Further development of this medium for use in fed batch culture in stirred bioreactors will be reported elsewhere.

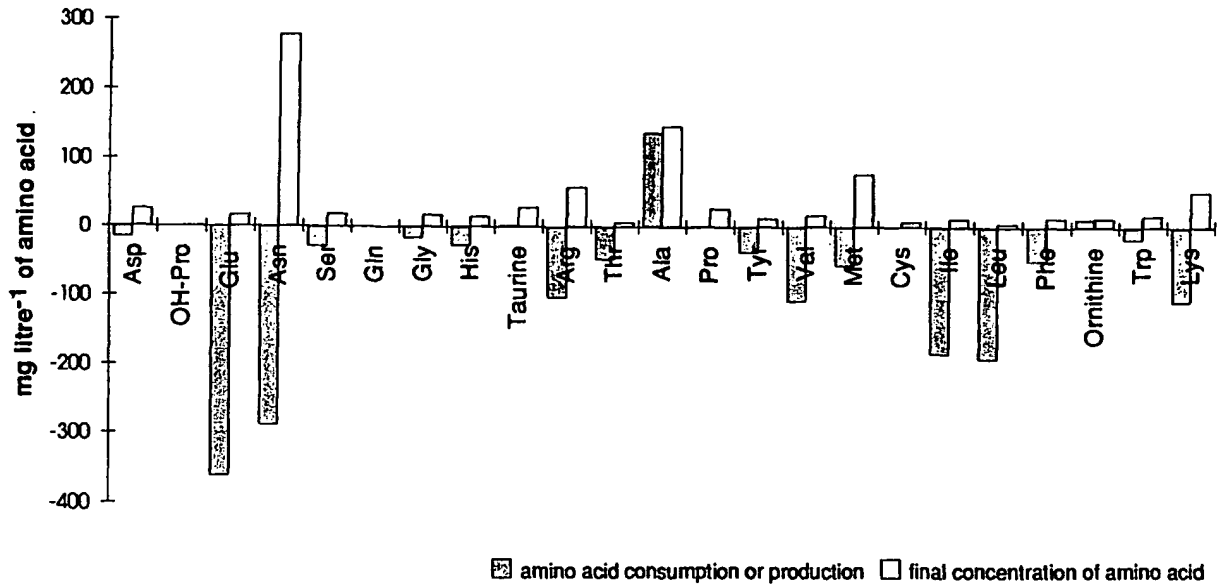


Fig. 3. Final concentration of amino acids and amino acid utilisation by NS0 9D4.CF cells cultured in shake flask using WNSD medium with recombinant insulin.

Antibody produced in serum-free medium is functionally active

CAMPATH-1H antibody produced in static or shake flask cultures in serum-containing or serum-free medium was examined for functional activity by ADCC. All preparations were found to have functional activity at 0.02 μg ml^{-1}. Further studies relating to the glycosylation and biological activity of CAMPATH-1H expressed in different cell lines and grown under different culture conditions will be published shortly (Lifely *et al.*, 1995).

Discussion

There are considerable advantages in the use of media that do not contain serum and that are essentially protein-free. In this study we have shown that GS-engineered NS0 cells expressing humanised monoclonal antibody can be grown in a novel protein-free medium, WNSA medium, supplemented with cholesterol rich lipid and β-cyclodextrin. WNSA is a complex medium of defined composition, containing a number of additional trace elements and vitamins. Poor growth of GS-engineered NS0 cell lines expressing humanised monoclonal antibody in serum-containing 'select' medium and WNSA suggested that these media were sub-optimal.

The nutritional requirements of NS0 cells are complicated by NS0 having an essential requirement for exogenous cholesterol. In this respect NS0 are similar to the NS-1 cell line that is deficient in 3-ketosteroid reductase activity and unable to convert lathosterol to cholesterol (Sato *et al.*, 1987). As NS0 are derived from NS-1 it is likely that they also lack 3-ketosteroid reductase activity. In serum, cholesterol and other lipids are found attached to carrier proteins such as LDL, HDL and albumin. The use of phosphatidylcholine:cholesterol vesicles with a carbohydrate carrier β-cyclodextrin enabled these cells to grow moderately well in protein-free medium. In order to overcome the complications caused by the cholesterol requirement, GS-engineered NS0 cells were adapted to cholesterol-independence. Several cloned GS-engineered NS0 and NS0 hybridoma lines secreting monoclonal antibody have also been adapted to cholesterol-independent growth (data not shown), with a 100% success rate. This implies that the majority, if not all, of individual cells within the NS0 cells population contain the genetic information required to produce active 3-ketosteroid reductase, but perhaps in a repressed form.

Nutrient depletion studies using cholesterol-requiring engineered NS0 cell lines in WNSA medium identified a number of amino acids that were being almost completely consumed. Interestingly the amino acid utilisation by the cholesterol-independent cell lines was found to be practically identical to that of the

lines prior to adaptation (data not shown), suggesting that amino acid metabolism was similar. An enriched medium (WNSD) was developed containing additional amino acids, choline chloride and ribonucleosides. Cholesterol-independent 9D4.CF cells grown in shake flask culture with WNSD reached a density of 1.86×10^6 cells ml^{-1} accumulating 76.6 mg $litre^{-1}$ functionally active (by ADCC) CAMPATH-1H antibody. In addition 9D4.CF reached a density of over 2.5×10^6 cell ml^{-1} in a one litre stirred bioreactor using WNSD supplemented with 0.1% w/v Pluronic F68 (data not shown). In WNSA medium it was found that human recombinant insulin (Nucellin) slightly improved cell growth, but that in WNSD Nucellin was not required. Amino acid analysis of spent WNSD culture supernatant showed that certain amino acids were still being fully depleted. A modification of WNSD medium which contained additional nutrients and a lower concentration of sodium salts (to adjust the osmolality) was toxic and inhibited cell growth. Future studies should concentrate on different feeding regimes to increase biomass, accumulated antibody and cell viability and on the prevention of cell death.

Acknowledgement

We would like to thank Pauline Rhodes for the electron microscopic examination which identified Apoptotic death of the cells and Dr J. Tite, Dr N. Rapson and Dr R. Lifely for there helpful discussions in the course of this work.

References

Bebbington CR, Renner G, Thomson S, King D, Abrams D and Yarranton GT (1992) High-level expression of recombinant antibody from myeloma cells using glutamine synthetase as an amplifiable selectable marker. Bio/Technology 10: 169–175.

Brown ME, Renner G, field RP and Hassel T (1992) Process Development for the production of recombinant antibodies using the glutamine Synthetase (GS) system. Cytotechnology 9(1–3): 231–236.

Dyer MJS, Hale G, Hayhoe FGJ, and Waldmann H (1989) Effect of CAMPATH 1 antibodies *in vivo* in patients with lymphoid malignancies: influence of antibody isotype. Blood 73: 1431–1439.

Galfre G and Milstein (1982) Chemical typing of human kappa light chain subgroups expressed by human hybrid myelomas. Immunology 45: 125–127.

Hale G, Clark M and Waldmann H. (1985) Therapeutic potential of monoclonal antibodies: isotype specificity of antibody-dependant cell-mediated cytotoxicity with human lymphocytes. J. Immunol. 134: 3056–3061.

Hale G, Cobbold SP, Waldmann H (1988a) T-cell depletion with Campath 1H antibodies in allogeneic bone marrow transplantation. Transplantation 45: 753–759.

Hale G, Dyer MJS, Clark MR, Philips JM, Marcus R, Reichmann L, Winter G, and Waldmann H (1988b) Remision induction in non-Hodgkins lymphoma with reshaped human monoclonal antibody, CAMPATH-1H. Lancet 2: 1394–1399.

Hale G, Xia M-Q, Tighe HP, Dyer MJS, and Waldmann H (1990) The CAMPATH-1H antigen (CDw52). Tissue Antigens 35: 118–127.

Isaacs JD (1990) The antiglobulin responce to therapeutic antibodies. Semin. Immunol 2: 449–456.

Isaacs JD, Watts RA, Hazleman BL, Hale G, Keogan MT Cobbold SP and Waldmann H (1992) Humanised monoclonal antibody therapy for rheumatoid arthritis. The Lancet 340 (Sept 26): 748–752.

Keen MJ (1995A) Development of a serum-free culture medium for the large scale production of recombinant protein from Chinese hamster ovary cells. Cytotechnology 17: 153–163.

Keen MJ (1995) High density culture of rat myeloma and rat hybridoma cells in a protein-free medium. Cytotechnology 17: 193–202.

Keen MJ and Steward T (1995) Adaptation of cholesterol-requiring NS0 mouse myeloma cells to high density growth in a fully defined protein-free and cholesterol-free culture medium. Cytotechnology 17: 203–211.

Lifely MR, Hale C, Boyce S, Keen MJ and Phillips J (1995) Glycosylation and biological activity of CAMPATH-1H expressed in different cell lines and grown under different culture conditions. Glycobiology 5(8): in press.

Nacheva E, Fischer P, Karpas A, Sherrington P, Hayhoe FGJ, Manolov G, Manolova Y, Ferstl G, Haas O, and Gadner H (1987) Complex translocation t (8;12;14) in a cell line derived from a child with nonendemic Burkitts-type acute lymphoplastic leukemia. Cancer Genet. Cytogenet.28: 145–153.

Sato JD. Kawamoto T and Okamoto T (1987) Cholesterol requirement of P3-X63-Ag8 mouse myeloma cells for growth in vitro. J. Exp. Med. 165: 1761–1766

Stryer L (1988)Biochemistry 3rd edition, W. H. Freeman and Co. Page 290.

Page MJ and Sydenham MA (1991) High level expression of the humanised monoclonal antibodiy Campath 1H in Chinese hamster ovary cells. Bio/Technology 9: 64–68.

Riechman L. Clark M, Waldman H and Winter G (1988) Reshaping human antibodies for therapy. Nature 332: 323–327.

Urlaub G and Chasin LA (1980) Isolation of Chinese hamster ovary cell mutants deficient in dihydrofolate reductase activity. Pros. Natl. Acad. Sci. USA 77(7):4216–4220.

Wallace GR (1994) Monoclonal antibodies as therapeutic agents. The Pharmaceutical Journal 252:715–718.

Winter G and Milstein C (1991). Man made antibodies. Nature 349:293–299. 24th Jan.

Address for correspondence: M. J. Keen, Biological Research Division, Wellcome Research Laboratories, Langley Court, Beckenham, Kent, UK.

0022-1767/91/14712-4366802.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 147, 4366–4373, No. 12, December 15, 1991
Printed in U.S.A.

CONSTRUCTION, EXPRESSION AND CHARACTERIZATION OF HUMANIZED ANTIBODIES DIRECTED AGAINST THE HUMAN α/β T CELL RECEPTOR

CLYDE W. SHEARMAN,[1]* DAN POLLOCK,* GARY WHITE,* KATHY HEHIR,* GORDON P. MOORE,[2]* E. J. KANZY,† AND ROLAND KURRLE†

*From the *Genzyme Corporation, Framingham, MA 01701; and †Behringwerke Aktiengesellschaft, D-3550 Marburg, West Germany*

Completely humanized antibodies with specificity for the human α/β TCR have been produced by genetic engineering. The L and H chain V region exons encoding the murine mAb BMA 031 CD regions and human EU framework regions were synthesized and replaced into previously isolated genomic fragments. These fragments were inserted into mammalian expression vectors containing the human κ and γ 1 C region exons. Two variants were constructed each containing selected BMA 031 amino acids within the human frameworks. The humanized genes were transfected into Sp2/0 hybridoma cells by electroporation and transfectomas secreting humanized antibody were isolated. Levels of antibody expression up to 7 pg/cell/24 h were obtained. The humanized antibody, BMA 031-EUCIV2, competed poorly with murine BMA 031 for binding to T cells. BMA 031-EUCIV3, however, bound specifically to T cells and competed effectively with both the murine BMA 031 antibody and a previously constructed chimeric BMA 031 antibody for binding to these cells. The relative affinity of BMA 031-EUCIV3 was about 2.5 times lower than BMA 031. The ability to promote antibody dependent cell-mediated cytolysis was significantly enhanced with the engineered antibodies as compared to murine BMA 031. Humanized BMA 031 is a clinically relevant, genetically engineered antibody with potential uses in transplantation, graft vs host disease, and autoimmunity.

mAb are emerging as a major modality for therapy of various pathologic conditions including malignant disease, cardiovascular disease, and autoimmune diseases. Some of these have demonstrated efficacy in treating colon carcinoma (1), B cell lymphomas (2), neuroblastoma (3), and in preventing transplant rejection (4, 5).

Clinical trials with murine antibodies, although encouraging, have indicated at least two fundamental problems of antibody therapy. First, murine IgG has a much shorter circulating half-life in man compared to what has been reported for human antibodies (6, 7), so that effective mAb therapy may require frequent multiple treatments with large amounts of murine antibody. Second, administration of murine IgG elicits a brisk HAMA[3] response that that can further reduce the circulating half-life of the mAb and produce allergic reactions including anaphylaxis (8–10).

Almost all of the murine mAb currently being used clinically provoke HAMA responses in patients. These include HAMA against both the C region and the V region (11). HAMA responses lead to altered pharmacokinetics of the injected mAb. The antibody is rapidly cleared from the serum and reduced antibody levels are attained (12). Although severe side effects are rare in patients with HAMA after retreatment with antibody, it is clear that if mAb are to be used therapeutically, reliable methods must be devised to reduce immune mediated complications or adverse reactions (13).

One approach to better immunotherapies currently being explored is to produce a truly human antibody. Unfortunately, human mAb technology has lagged far behind that of murine-based monoclonal technology. Human hybridomas are difficult to prepare, are often unstable, and secrete antibody at low levels (14, 15). The mAb generated are usually of the IgM class and of low affinity.

An attractive and viable strategy is to produce "humanized" versions of murine mAb through genetic engineering. Methods have been devised to replace all regions of a murine antibody with analogous human regions (16–18). Chimeric antibody technology has been applied to several therapeutically important antibodies (19–24) and has been useful in class switching and the production of isotypes with specific effector functions (25, 26). A chimeric antibody composed of the V regions of murine mAb 171A and the human γ 1C region has recently been used in patients with colon cancer. Whereas murine 171A has been used extensively in clinical trials and elicits a very pronounced HAMA response that alters its pharmacokinetics, antibody responses to chimeric 171A have been dramatically reduced. Moreover, the circulating half-life was increased relative to murine 171A and higher serum levels could be maintained at lower infused doses (27). Thus, with judicious genetic engineering, it is possible to manipulate antibody pharmacokinetics to minimize toxic side effects.

Chimeric antibodies may be effective in lowering the HAMA response in patients and increasing serum half-lives, but these properties are still inferior to human

Received for publication March 18, 1991.
Accepted for publication September 16, 1991.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and reprint requests to Dr. C. W. Shearman, Sterling Drug, Inc., 512 Elmwood Avenue, Sharon Hill, PA 19079.
[2] Present address Department of Molecular Genetics, SmithKline Beecham, 709 Swedeland Road, King of Prussia, PA 19406.

[3] Abbreviations used in this paper: HAMA, human anti-murine antibody; ADCC, antibody dependent cell mediated cytolysis; FR, framework region.

antibodies. Inasmuch as chimeric antibodies are still 30% murine, enhanced efficacy may be obtained by humanizing the V regions. New technologies have recently been advanced to produce totally humanized antibodies by grafting the CDR of murine antibodies onto human FR (17, 28–30). The resulting antibodies when expressed with human C regions should be essentially human. This technology, although technically straightforward, is not always totally successful. Selected FR amino acids appear to be involved in Ag binding. Identification of important FR amino acids has been achieved, up to now, by the use of x-ray crystallographic data (17) and sophisticated computer modeling (30) and several totally humanized antibodies have been produced with affinities close to those of their parental antibodies (17, 28–30).

We report here the production of a humanized antibody, without the use of sophisticated structural data, which retains the affinity and specificity of BMA 031, a murine mAb directed against the human α/β TCR. Moreover, humanized BMA 031 displays enhanced ADCC activity. BMA 031 has been used successfully in preventing organ transplant rejection (5) and may have potential efficacy in other T cell-related disorders.

MATERIALS AND METHODS

Cell culture. The BMA 031 and Sp2/0-Ag14 hybridomas were cultured in DMEM media supplemented with 10% FCS, 2 mM L-glutamine, 10 mM HEPES, pH 7.3, 10 mM nonessential amino acids (GIBCO, Gaithersburg, MD), and 10 mM pyruvate. Chimeric and humanized BMA 031 transfectomas were grown in the above media containing 1 μg/ml mycophenolic acid, 50 μg/ml xanthine, and 500 μg/ml Geneticin (GIBCO). All lines were maintained at 37°C in 7% CO_2.

Computer analysis. Sequences were manipulated and homology searches were performed with the Genetics Computer Group Sequence Analysis Software Package (University of Wisconsin Biotechnology Center, Madison, WI)) using the National Biomedical Research Foundation databases.

Synthesis of VH and VL regions. The VH and VL exons were synthesized on an Applied Biosystems (Foster City, CA) model 380A DNA synthesizer. Each region was synthesized completely as *Eco*RI-*Hin*dIII fragments consisting of overlapping (10–15 nucleotide overlap) oligomers (75–110 nucleotides). The oligomers were deprotected and purified by electroelution from polyacrylamide gels. The oligomers were then mixed in equimolar amounts (30 pmol), phosphorylated, annealed, and ligated into pUC 19 previously digested with *Eco*RI and *Hin*dIII.

Nucleotide sequencing. DNA sequencing of the synthesized VH and VL regions was performed directly on pUC subclones using universal forward and reverse primers (31).

Construction of humanized genes. To ensure efficient expression, the synthesized V regions were inserted into previously isolated genomic fragments (24) in place of the murine V regions. The resulting 5.6-kb *Eco*RI VH fragment was cloned into a mammalian expression vector containing the human γ1 C region and the *gpt* gene for selection. The 3.0-kb *Hin*dIII VL fragment was cloned into a vector containing the human κ C region and the *neo* gene (see Fig. 5).

Transfection of DNA into Sp2/0 cells by electroporation. DNA was introduced into murine hybridoma Sp2/0-Ag14 cells by electroporation. The 1 to 2×10^7 actively growing Sp2/0-Ag14 cells were washed and resuspended in 1.0 ml of sterile PBS. A total of 15 μg of each humanized, Igκ and IgG1, plasmid (linearized with *Bam*HI) was added to the cell suspension. The DNA/cells were transferred to a precooled shocking cuvette, incubated on ice at least 5 min and then a 0.5 kv/cm electric pulse was delivered for 10 ms (Transfector 300, BTX, San Diego, CA). After shocking, the DNA/cell mixture was returned to ice for 10 min and then diluted in 40 ml of supplemented DMEM and incubated at room temperature for 10 min. Finally, the cells were transferred to a 37°C incubator with 7% CO_2 for 48 h before plating in selective medium, containing 1 μg/ml mycophenolic acid, 50 μg/ml xanthine, and 1 mg/ml Geneticin. Cells were plated in 96-well plates at 3×10^4 cells/well.

Cytofluorometric assay for affinity. To analyze the relative affinities of murine, chimeric, and humanized BMA 031 antibodies, competitive immunofluorescence assays were carried out. PBMC were separated by Ficoll-Hypaque density gradient centrifugation and incubated on ice for 1 h in the dark with mAb at various concentrations (0.05–50 μg/ml) premixed with either FITC-BMA031 or FITC-BMAEUCIV3 (2 μg/ml). Unbound antibodies were removed by two washing steps. Cells from all experiments were analyzed either on an Ortho (Raritan, NJ) Cytofluorograph 50H/2150 Computer System or on a Becton Dickinson (Mountain View, CA) FACStar Plus as described elsewhere (32). The intensity of fluorescence was calculated by modified Ortho or standard FACStar Plus software and is expressed as mean channel number.

Cytotoxicity assays. To measure the cytolytic capacity of the BMA 031 antibody preparations, a 20 h [^{51}Cr] release assay was performed to measure ADCC and NK activity. [^{51}Cr]-labeled HPB-ALL target cells were incubated with (ADCC) or without (NK activity) various concentrations of antibodies for 20 h in the presence of Ficoll-separated PBL (effector cells). α/β TCR negative CEM cells were used as control target cells. The antibodies were allowed to bind first to target cells (30 min) before the effector cells were added. The E:T cell ratio varied from 1:1 to 50:1. Cytolysis in the absence of antibodies was considered to be due to NK activity. The percentage of specific lysis was calculated as described earlier (33). Spontaneous [^{51}Cr] release in the absence of effector cells and in the presence of the antibodies being tested was always less than 5%. All samples were analyzed in triplicate.

RESULTS

Designing humanized BMA 031 antibodies. To determine the optimal human sequence with which to humanize the murine BMA 031 antibody, the murine BMA 031 amino acid sequence was used to search the NBRF data base for the most homologous human antibody. Inasmuch as molecular models of antibodies show strong interactions between the H and L chains, we decided to use the H and L chain from the same human antibody. The human EU antibody turned out to be the best overall choice. The homology between the BMA 031 and EU FR (nos. 1–3) was 79% (67% identical) for the H chain and 81% (63% identical) for the L chain. The BMA 031 antibody uses JH3 and JK5. These are most homologous to human JH4 and JK4. A first generation humanized BMA 031 antibody would contain BMA 031 CDR, EU FR, and homologous human J regions. We refer to this antibody as BMA 031-EUCIV1 (Fig. 1).

A refinement to this basic humanized version can be made in the sequence immediately before and after the CDR. The CDR are assigned based on sequence homology data (34). Molecular models of antibodies have shown that the actual CDR loops can contain amino acids up to five amino acids away from the "Kabat" CDR (36). Also, Reichmann et al. (17) have shown the functional importance of a FR amino acid four residues from a CDR. Therefore, maintaining at least the major amino acid differences (in size or charge) within four amino acids of the CDR as murine may be beneficial. We refer to the antibody containing these changes as BMA 031-EUCIV2 (Fig. 1). Additionally, all differences within four amino acids of the CDR could be maintained murine. We refer to this antibody as BMA 031-EUCIV3.

Further refinements can be made, but, without complex computer modeling, it is difficult to prioritize their importance. For example, several amino acids are either BMA 031 specific or EU specific (i.e., different from the consensus sequence within their subgroups). Inasmuch as these amino acids presumably arose through somatic mutation to enhance their respective activities, it would seem logical to maintain the BMA 031-specific amino acids and change the EU-specific amino acids to the human consensus. But this can have potential adverse

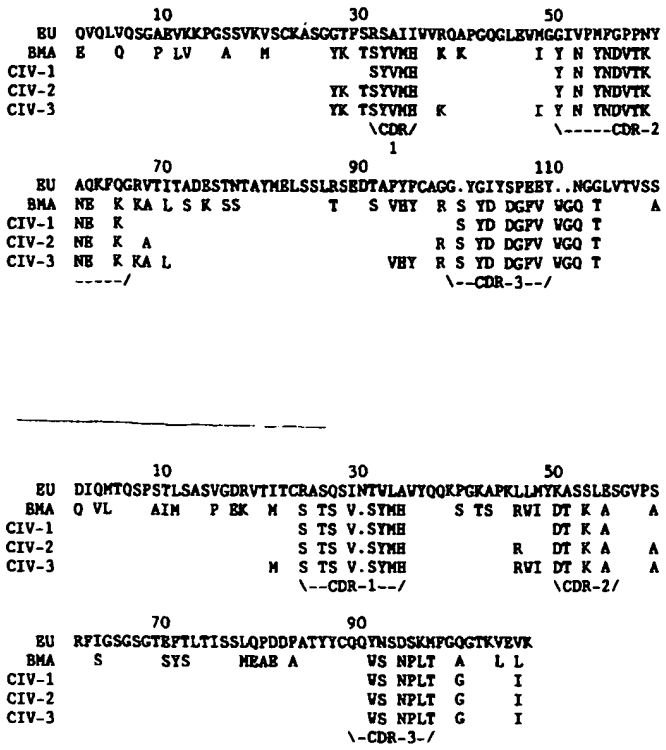


Figure 1. Amino acid sequences of EU, BMA 031, and humanized BMA 031 V regions. *A*, The VH region and *B*, the VL region. The positions of the CDR are indicated.

TABLE I

Amino acid (AA) differences between BMA 031 and EU and their consensus sequences

AA Position[a]	EU AA	Human AA	BMA031 AA	Mouse AA
H chain, EU specific				
70	Ile	[b]	Leu	Leu
72	Ala	[b]	Ser	Val
74	Glu	[b]	Lys	Lys
93	Phe	Val	Val	Val
95	Phe	Tyr	Tyr	Tyr
98	Gly	Arg	Arg	Arg
L chain, EU specific				
10	Thr	Ser	Ile	Ile
48	Met	Ile	Ile	Ile
63	Ile	Ser	Ser	Ser
70	Glu	Asp	Ser	Ser
81	Asp	Glu	Glu	Glu
H chain, BMA specific				
1	Gln	Gln	Glu	Gln
7	Ser	Ser	Ser	Pro
9	Ala	Ala	Pro	Ala
20	Val	Val	Met	Leu
40	Ala	Ala	Lys	Arg
72	Ala	[b]	Ser	Val
82	Glu	Glu	Glu	Gln
94	Tyr	Tyr	His	Tyr
L chain, BMA specific: None				

[a] Numbers correspond to those in Figure 1.
[b] Variable.

consequences. Changing an amino acid in one chain may cause changes in the interactions with other amino acids of that chain as well as with amino acids in the other chain. Therefore, extreme caution must be exercised to limit the number of changes. Table I outlines these potential changes. The residue numbers correspond to those in Figure 1. As can be seen, EU differs from the human VH-I subgroup consensus sequence in six positions. Three are within four amino acids of the CDR (nos. 70, 95, and 98), and these are addressed in BMA-031-EUCIV3. In one position (no. 93) the human consensus sequence is the same as BMA 031. Moreover, the Phe_{93} in EU is highly unusual; this amino acid is only found in this position in one other human antibody in subgroup VH-III. One could rationalize changing this from EU to the human consensus, so we incorporated this change into BMA 031-EUCIV3. For the two remaining positions (nos. 72 and 74), there is no clear human consensus so we maintained the EU sequence. The L chain had five EU-specific amino acids. One is within four amino acids of the CDR (no. 48) and is maintained as BMA 031 in BMA 031-EUCIV3. In two positions (nos. 63 and 81) the human consensus is the same as BMA 031 and therefore could be changed to the human consensus. We decided not to make these changes at this time. The other two positions (nos. 10 and 70) were also not changed to limit the number of substitutions. There are eight BMA 031 specific amino acids in the H chain. In two positions (nos. 7 and 82) the BMA 031 sequence is the same as EU. His_{94} is unique to BMA 031. This position is considered "invariant" with Tyr_{94} occurring more than 98% of the time. Therefore, we decided to incorporate this change into BMA 031-EUCIV3. The remaining five positions (nos. 1, 9, 20, 40, and 72) were maintained EU to limit the number of changes. There are no BMA 031-specific amino acids in the L chain. The sequence is identical to the subgroup VI consensus. The changes in the human EU framework sequence back to BMA 031 are summarized in Table II. Twelve changes were made in the H chain; 5 in BMA 031-EUCIV2 and 7 more in BMA 031-EUCIV3. Five changes were introduced into the L chain; two in BMA 031-EUCIV2 and three more in BMA 031-EUCIV3.

Determination of DNA sequence for humanized V regions. The amino acid sequence of the V regions were reverse translated using the actual BMA 031 codons wherever possible and BMA 031 codon preferences everywhere else. To aid in future modifications, unique restriction enzyme sites were engineered into the sequence at approximately 60-bp intervals by making use of the degeneracy of the genetic code. Finally, convenient restriction enzyme sites 5′ and 3′ of the coding region of BMA 031 were identified and this flanking sequence was incorporated into the final humanized sequence to be syn-

TABLE II

Amino acid (AA) changes in EU FR

AA Position[a]	EU AA	BMA031 AA	CIV2 AA	CIV3 AA
H chain				
27	Gly	Tyr	Tyr	Tyr
28	Thr	Lys	Lys	Lys
30	Ser	Thr	Thr	Thr
38	Arg	Lys	Arg	Lys
48	Met	Ile	Met	Ile
67	Arg	Lys	Arg	Lys
68	Val	Ala	Ala	Ala
70	Ile	Leu	Ile	Leu
93	Phe	Val	Phe	Val
94	Tyr	His	Tyr	His
95	Phe	Tyr	Phe	Tyr
98	Gly	Arg	Arg	Arg
L chain				
21	Ile	Met	Ile	Met
46	Leu	Arg	Arg	Arg
47	Leu	Trp	Leu	Trp
48	Met	Ile	Met	Ile
60	Ser	Ala	Ala	Ala

[a] Numbers correspond to those in Figure 1.

thesized. The final DNA sequences of BMA 031-EUCIV2 VH and VL, excluding the *Eco*RI and *Hin*dIII cloning ends, are shown in Fig. 2.

Synthesis of humanized BMA 031 V regions. The L and H chain V region exons encoding the humanized antibodies were synthesized completely as *Eco*RI-*Hin*dIII fragments consisting of 10 to 15 overlapping (10–15 nucleotide overlap) oligomers (75–110 nucleotides). The oligomers were phosphorylated, annealed and ligated into a pUC vector previously cut with *Eco*RI and *Hin*dIII. The assembled fragments were sequenced to verify accuracy of synthesis.

Reconstruction of BMA 031 genomic fragments with humanized V exons. To increase the probability of efficient expression of the synthesized coding regions, the humanized sequences were replaced into the previously isolated 5.6-kb *Eco*RI VH and 3.0-kb *Hin*dIII VL genomic fragments of BMA 031 (Fig. 3). Due to the lack of unique restriction enzyme sites, several subclonings were necessary. To achieve this goal, four vectors, each containing modified genomic subfragments, were constructed. The first vector, pUCBMAVH-1.0HΔN was constructed by subcloning the 1.0-kb *Hin*dIII BMA 031 VH fragment into pUC19 with subsequent deletion of the 5′-*Nsi*I site. The second vector, pUCΔHBMAVH-5.6RΔH, was derived by cloning the 5.6-kb *Eco*RI BMA 031 VH fragment into a pUC19 vector with a previously deleted *Hin*dIII site. The 5′-*Hin*dIII site of the insert was then deleted to complete the construction. The third vector, pUCBMAVL-1.4RH2, was constructed by subcloning the 1.4-kb *Eco*RI-*Hin*cII BMA 031 VL fragment into pUC19. The fourth vector, pUCΔRSBMAVL-3.0H, was made by cloning the 3.0-kb *Hin*dIII BMA 031 VL fragment into a pUC19 vector that had a previous deletion from the *Eco*RI site to the *Sal*I site in the polylinker.

The cloning scheme to replace the humanized sequences into the genomic fragments is outlined in Figure 4. The newly synthesized *Sau*I-*Nsi*I BMA 031-EUCIV2 VH fragment was isolated from the pUC19 subclone and cloned into pUCBMAVH-1.0HΔN. Then, the 1.0-kb *Hin*dIII fragment was isolated and cloned into pUCΔHBMAVH-5.6RΔH. Finally, the 5.6-kb *Eco*RI fragment was isolated and subcloned into the mammalian expression vector containing the human γ 1 C region and the *gpt* gene for selection (Fig. 5).

The newly synthesized *Sau*I-*Hin*cII BMA 031-EUCIV2 VL fragment was isolated and cloned into pUCBMAVL-1.4RH2. Then, the 1.4-kb *Eco*RI-*Hin*cII fragment was isolated and cloned into pUCΔRSBMAVL-3.0H. Finally, the 3.0 *Hin*dIII fragment was isolated and cloned into the mammalian expression vector containing the human κ C region and the *neo* gene for selection (Fig. 5).

The BMA 031-EUCIV3 constructs were prepared in the same manner as BMA 031-EUCIV2. Replacement oligomers incorporating the coding changes for BMA 031-EUCIV3 were synthesized and cloned into the pUCBMA-EUCIV2 constructs. The final clone was sequenced to ensure accuracy of the coding sequence. The BMA 031-EUCIV3 V regions were replaced into the original BMA 031 genomic fragments and these fragments were cloned into the mammalian expression vectors described above.

Expression and purification of humanized BMA 031 antibodies. The humanized genes were transfected into Sp2/0 hybridoma cells by electroporation and selected in media containing both mycophenolic acid and Geneticin. Transfectomas secreting humanized BMA 031 antibodies were identified by ELISA. Secretion levels up to 7 pg/cell/24 h were obtained. The best clone from each transfection (CIV2 and CIV3), with respect to secretion level and growth characteristics, was expanded for further study.

The BMA 031-EUCIV2 and -EUCIV3 antibodies were partially purified by protein A-Sepharose column chromatography. Analysis of the antibodies by reducing and nonreducing SDS-PAGE showed a high degree of purity (data not shown). Analysis by a series of ELISA assays showed that the antibodies contained human κ and γ 1 C

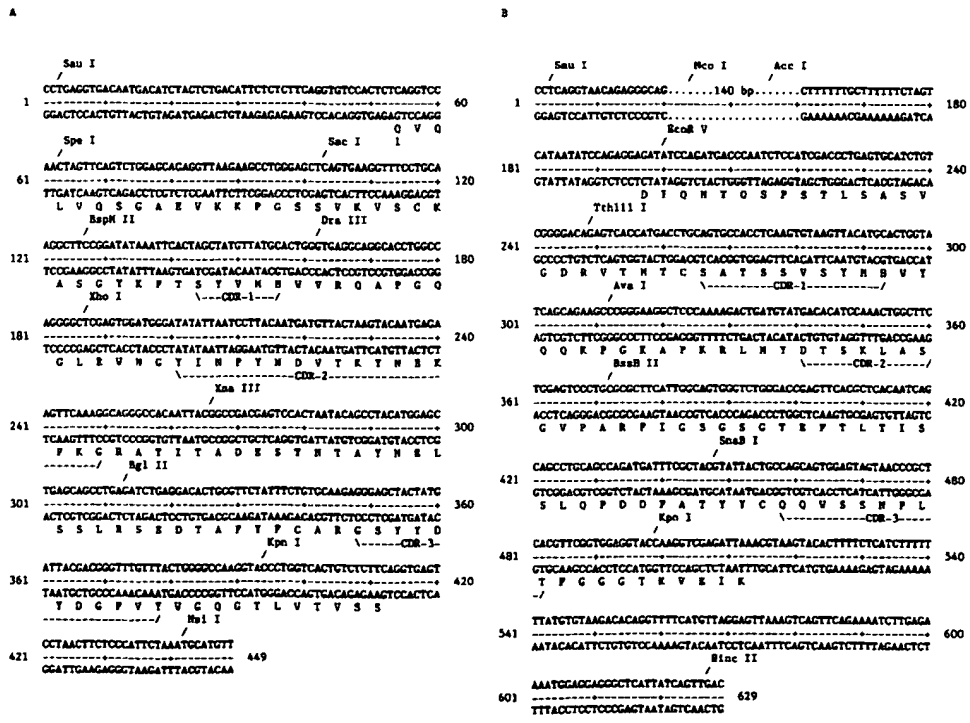


Figure 2. DNA sequences of the V regions of BMA 031-EUCIV2. *A*, The BMA 031-EUCIV2 VH region and *B*, the BMA 031-EUCIV2 VL region.

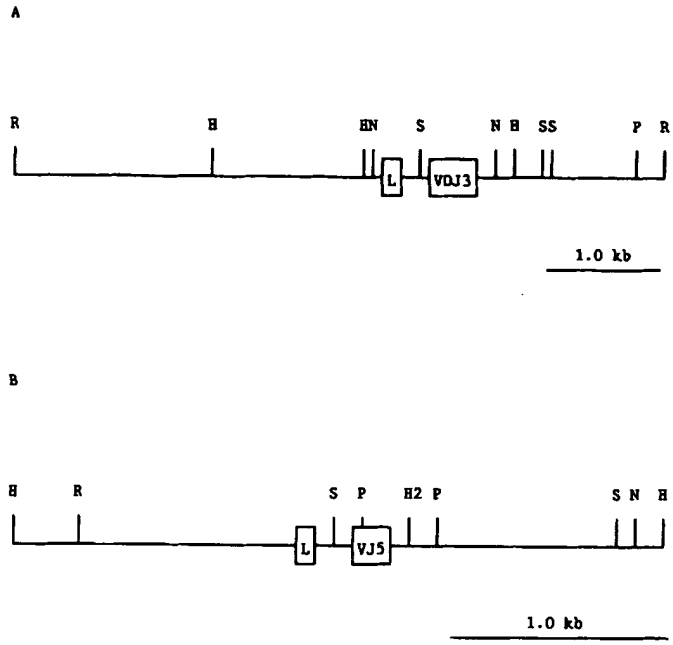


Figure 3. Partial restriction enzyme maps of BMA 031 V regions. *A*, The 5.6-kb *Eco*RI VH fragment containing the VDJ_3 exon. *B*, the 3.0-kb *Hin*dIII VL fragment containing the VJ_5 exons. *H*, *Hin*dIII; *H2*, *Hin*cII; *N*, *Nsi*I; *P*, *Pst*I; *R*, *Eco*RI; *S*, *Sau*I.

regions. Moreover, the antibodies did not react with anti-murine antibodies (data not shown).

Characterization of humanized BMA 031 antibodies. The BMA 031-EUCIV2 antibody bound poorly to T cells. In contrast, BMA 031-EUCIV3 shows an identical specificity as murine BMA 031. They both bind specifically to T cells and show no reactivity toward monocytes, E, or granulocytes (data not shown).

The relative affinities of murine BMA 031, chimeric BMA 031 (human IgG1), and the humanized variants were compared by competitive immunofluorescence assays. The data shown in Figure 6 indicate that both the murine BMA 031 antibody and the previously constructed chimeric BMA 031-G1 antibody block the binding of BMA 031-FITC in the same dose-dependent manner. BMA 031-EUCIV3 was about 2.5 times less efficient than murine BMA 031. BMA 031-EUCIV2 was unable to totally block BMA 031-FITC binding, even at concentrations as high as 50 μg/ml.

BMA 031 has been shown to be poor at mediating ADCC using human effector cells. To evaluate the ADCC capacity of the humanized antibodies, we compared them to rabbit anti-GH-1 antiserum. This antiserum was the best of eight rabbit anti-human T cell globulins in ADCC capacity. As shown in Figure 7, both the chimeric BMA 031 antibody and the BMA 031-EUCIV3 antibody were very efficient at ADCC. Even at very low effector:target cell ratios (Fig. 7*A*) or extremely low antibody concentrations (Fig. 7, *B* and *C*), the engineered antibodies are highly potent at mediating killing of the HPB-ALL cells.

DISCUSSION

We have joined the DNA segments containing the CDR from the BMA 031 mAb specific for the α/β TCR and the FR from the human EU antibody to the DNA segments encoding human γ-1 and κ C regions. When the humanized genes were introduced into non-Ig producing Sp2/0 cells, functional humanized antibodies specific for T cells were assembled and secreted.

Functional antibody, however, was dependent on substitution of various murine FR amino acids into the human FR. The identification of important FR amino acids in the absence of structural data or computer models is difficult but, by careful analysis of antibody sequence homologies, it is possible to generate a humanized sequence with a high probability of maintaining Ag binding. Our method consists of three parts. First, and possibly most important, is starting with the human antibody most homologous to the murine antibody under

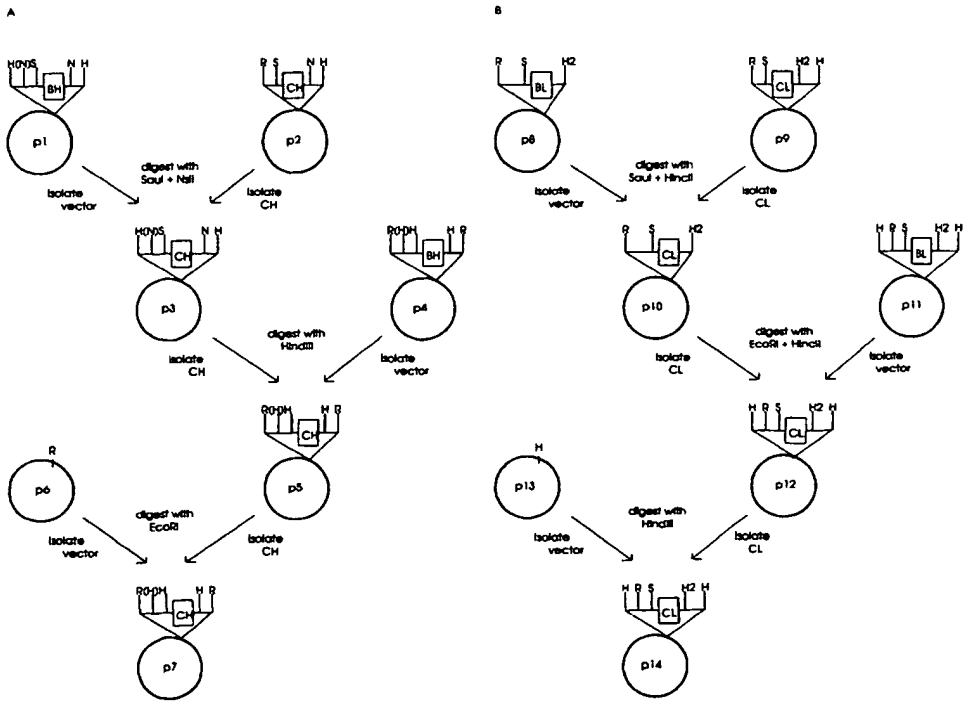


Figure 4. The cloning scheme to regenerate the BMA 031 genomic fragments with the humanized V regions. *A*, Substituting the humanized VH region into the 5.6-kb *Eco*RI VH fragment. *BH*, BMA 031 VH exon; *CH*, humanized BMA 031 VH exon; *p1*, pUCBMAVH-1.0HΔN; *p2*, pUCBMACIVH; *p3*, pUCBMACIVH-1.0HΔN; *p4*, pUCΔHBMAVH-5.6RΔH; *p5*, pUCΔHBMACIVH-5.6RΔH; *p6*, pSV2gpt-huγ1; *p7*, pSV2gpt-BMACIVH-huγ1. *B*, Substituting the humanized VL region into the 3.0 *Hin*dIII VL fragment. *BL*, BMA 031 VL exon; *CL*, humanized BMA 031 VL exon; *p8*, pUCBMAVL-1.4RH2; *p9*, pUCBMACIVL; *p10*, pUCBMACIVL-1.4RH2; *p11*, pUCΔRSBMAVL-3.0H; *p12*, pUCΔRSBMACIVL-3.0H; *p13*, pSV2neo-huκ; *p14*, pSV2neo-BMACIVL-huκ. Restriction enzyme sites identified are: *H*, *Hin*dIII; *H2*, *Hin*cII; *N*, *Nsi*I; *R*, *Eco*RI; *S*, *Sau*I.

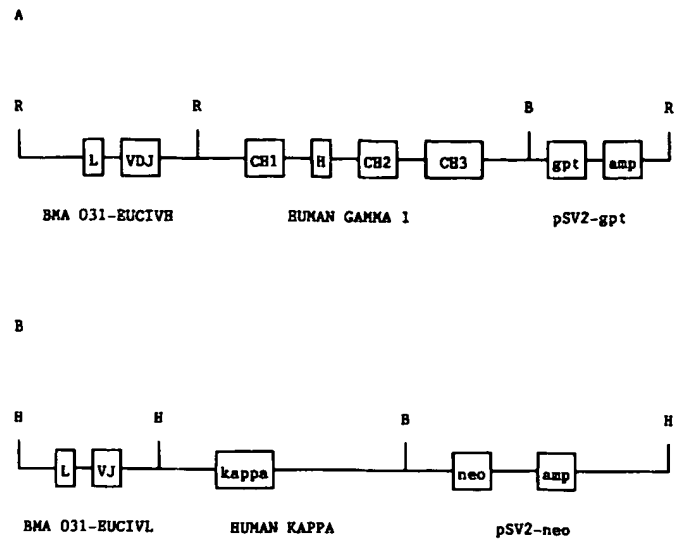


Figure 5. Expression vectors for humanized BMA 031 V regions. *A*, The H chain expression vector containing the humanized BMA 031 VH region, the human γ 1 C region, and the guanine phosphoribosyl transferase gene for selection. *B*, The L chain expression vector containing the humanized BMA 031 VL region, the human κ C region and the neomycin resistance gene for selection.

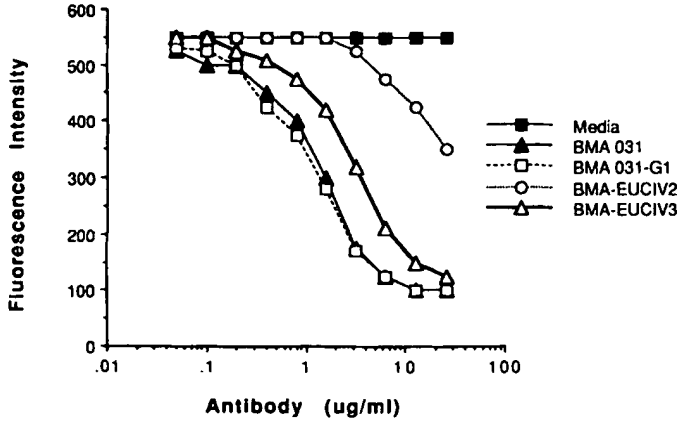


Figure 6. Relative affinities of BMA 031 antibodies. Competitive immunofluorescence assays with the BMA 031 antibodies, HPB mononuclear cells and BMA 031-FITC (2 μg/ml) were performed as outlined in *Materials and Methods*. Intensity of fluorescence is expressed as mean channel number.

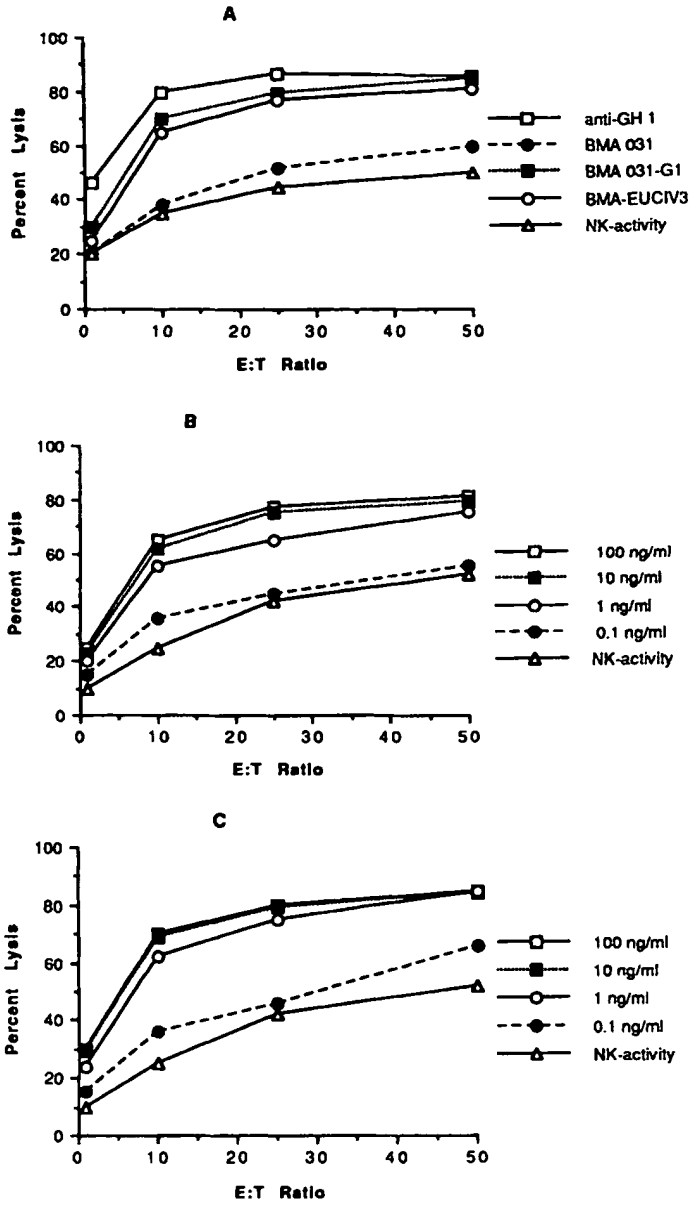


Figure 7. ADCC capacity of BMA 031 antibodies. The cytolytic capacity of the BMA 031 antibodies was determined in a 20 h [^{51}Cr] release assay as described in *Materials and Methods*. *A*, Lysis in the presence (ADCC) or absence (NK activity) of antibody (100 ng/ml). *B*, Lysis at various concentrations of BMA 031-EUCIV3. *C*, Lysis at various concentrations of chimeric BMA 031.

study. This effectively limits the number of amino acid differences that must be addressed. Second, because the assignment of CDR is based on homology and not function, the choice of maintaining the murine sequence on either side of the CDR is important. Evidence is emerging that the "functional" CDR loops can be displaced from the "Kabat" CDR by as many as five amino acids. Kabat et al. (34) places CDR-1 of the H chain V region from amino acids nos. 31 to 35 whereas crystal structure shows the loop to be from residues nos. 26 to 32 (35). Third, the identification of potentially "Ag specific" amino acids in both the human and murine antibody may be important. Although the identification may be straightforward, prioritizing their importance is very difficult. Inasmuch as the goal is to produce the most human-like sequence, these changes must be kept at a minimum. Our decision to keep similar amino acids human and only change the more unusual amino acids turned out to be correct in this instance. However, in the event that the humanized antibody was not functional, this analysis provides insight into those amino acids that could be altered to regain activity.

The T cell binding data with the humanized BMA 031 antibodies show the importance of FR amino acids in Ag binding. Inclusion of only the BMA 031 CDR (BMA 031-EUCIV1) would, most likely, not have been sufficient to maintain affinity for Ag. Twelve amino acid substitutions were made in the H chain V region to regain binding affinity (nos. 27, 28, 30, 38, 48, 67, 68, 70, 93, 94, 95, and 98). Of these, six may be more important (nos. 38, 48, 70, 93, 94, and 95) because they represent changes from BMA-EUCIV2, which does not bind well, to BMA 031-EUCIV3, which does bind well. Similarly, for the L chain V region, five amino acid substitutions were made (nos. 21, 46, 47, 48, and 60). Of these, three (nos. 21, 47, and 48) were made from BMA 031-EUCIV2 to BMA 031-EUCIV3 and thus may be more important.

The importance of any or all of these changes is difficult to assess. However, some of these changes appear to be quite significant. Five amino acid substitutions in the VH region stand out. Residues 70, 95, and 98 are both EU specific and fall within four amino acids of the CDR. Phe_{93} in EU is highly unusual. Only one other human antibody had this residue in this position. His_{94} in BMA 031 is also unusual. This is normally an invariant Tyr_{94}. In the L chain V region, residue 48 may be significant because it is both EU specific and within four amino acids of CDR-2.

Simple CDR replacement appears to be rarely successful, especially for antibodies directed against protein Ag. The initial attempt at humanizing an antibody involved only the H chain, used homologous human FR, and used antibody and Ag with solved crystal structures. The humanized antibody bound Ag about 10-fold less efficiently than the original murine antibody (29). The latest attempt to CDR-replace an antibody, reported to date, involved both the H and L chains of CAMPATH-1 (17). The resulting humanized antibody bound the CAMPATH-1 Ag very weakly. In both cases, the loss of binding activity was due to a single amino acid substitution in FR-1 of the H chain.

As shown above, simply replacing CDR sequences are not sufficient to ensure Ag-antibody binding. FR sequences obviously play a role in antibody affinity. The FR-1 amino acid identified above, Phe-27, was conspicuously missing from the human NEWM antibody. This alteration may have been important for NEWM activity but not for the activities of the other antibodies. It is interesting to note that Phe-27 is found in most human antibodies. This shows the importance of amino acid substitutions in particular antibody reactivities.

A study analyzing spontaneous variants of an antidigoxin antibody-producing hybridoma demonstrated the importance of FR-3 amino acids. A single amino acid change at residue 94 of the H chain V region caused a reduction in affinity from 5.4×10^9 to 9.2×10^6 M^{-1} (36). Spontaneous variant antibodies with reduced or absent Ag binding were also reported for phosphocholine and 4-hydroxy-3-nitro-5-iodophenyl-acetyl haptens (37–41). In these cases the structural changes were due to point mutations in either CDR or FR. A recent study by Queen et al. (30) used computer modeling to humanize the anti-Tac mab. They identified 10 potentially important FR amino acids that, when substituted into the final humanized sequence, yielded functional antibody. These results show the importance of FR amino acids on antibody affinity and the need for more detailed studies identifying the important sites.

Simple CDR replacement would only be successful, most likely, for antibodies that have undergone minimal affinity maturation. This would apply to both the murine and human antibodies. If affinity maturation involved the FR, then it becomes imperative to identify the "Ag specific" amino acids in the two antibodies. Perhaps using a germline human sequence or a human consensus sequence would be the best starting point for humanization. Then one would need only identify the important murine FR amino acids to include in the final sequence.

From the results presented, humanized BMA 031 may have clinical utility in preventing transplant rejection, graft vs host disease, autoimmune diseases, and other T cell-related problems. The humanized antibody retains the affinity and specificity of murine BMA 031 but contains humanized V regions and human C regions that should eliminate the immune response in patients during immunotherapy. Also, effector functions associated with the human C regions are enhanced in vitro relative to murine BMA 031 and may also be in vivo when the humanized antibody is used for immunotherapy. We are now in a position to test the efficacy of humanized BMA031 in clinical trials.

It seems clear from the growing body of evidence that a fully engineered antibody would reduce immunogenicity to a minimum, increase circulating half-life and enhance effector functions; and as such would constitute, from both safety and efficacy considerations, a preferred form of a new antibody at the onset of the first human clinical trials.

Acknowledgment. We thank P. Thammana, D. Barone, and L. Sun for helpful discussions and K. Frank, W. Lang, and W. Seyfert-Brandt for expert technical assistance.

REFERENCES

1. **Sears, H. F., D. Herlyn, Z. Steplewski, and H. Koprowski.** 1985. Phase II clinical trial of a murine monoclonal antibody cytotoxic for gastrointestinal adenocarcinoma. *Cancer Res. 45:5910.*
2. **Lowder, J. N., T. C. Meeker, M. Campbell, C. F. Garcia, J. Gralow, R. A. Miller, R. Warnke, and R. Levy.** 1987. Studies on B lymphoid tumors treated with monoclonal anti-idiotypic antibodies: correlation with clinical responses. *Blood 69:199.*
3. **Cheung, N. -R. V., H. Lazarus, F. D. Miraldi, C. R. Abramowski, S. Kallick, U. M. Saarinen, T. Spitzer, S. E. Strandjord, R. F. Coccia, and N. A. Berger.** 1987. Ganglioside G2 specific monoclonal antibody 3F8: phase I study in patients with neuroblastoma and malignant melanoma. *J. Clin. Oncol. 5:1430.*
4. **Goldstein, G., J. Schindler, H. Tsai, A. B. Cosimi, P. Russell, and D. Norman.** 1985. A randomized clinical trial of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. *N. Engl. J. Med. 313:337.*
5. **Kurrle, R., K. H. Enssle, and F. R. Seiler.** 1988. Monoclonal antibodies directed to leukocyte differentiation antigens for therapeutic use. *Behring Inst. Mitt. 82:154.*
6. **LoBuglio, A. F., M. Saleh, L. Peterson, R. Wheeler, R. Carrano, W. Huster, and M. B. Khazaeli.** 1986. Phase I clinical trial of CO17-1A monoclonal antibody. *Hybridoma 5:117.*
7. **Morrell, A., W. D. Terry, and T. A. Waldmann.** 1970. Metabolic properties of IgG subclasses in man. *J. Clin. Invest. 49:673.*
8. **Shawler, D. L., R. M. Bartholomew, L. M. Smith, and R. O. Dillman.** 1985. Human immune response to multiple injections of murine monoclonal IgG. *J. Immunol. 135:1530.*
9. **Sears, H. F., D. Herlyn, Z. Steplewski, and H. Koprowski.** 1984. Effects of monoclonal antibody immunotherapy on patients with gastrointestinal adenocarcinoma. *J. Biol. Response Mod. 3:138.*
10. **Miller, R. A., A. R. Oseroff, P. T. Stratte, and R. Levy.** 1983. Monoclonal antibody therapeutic trials in seven patients with T-cell lymphoma. *Blood 62:988.*
11. **Jaffers, G. J., T. C. Fuller, A. B. Cosimi, P. S. Russell, H. J. Winn, and R. B. Colvin.** 1986. Monoclonal antibody therapy—Anti-idiotypic and non-anti-idiotypic antibodies to OKT3 arising despite intense immunosuppression. *Transplantation 41:572.*
12. **Khazaeli, M., M. Saleh, R. Wheeler, W. Huster, H. Holden, R. Carrano, and A. LoBuglio.** 1988. Phase-1 trial of multiple large doses of murine monoclonal antibody Co17-1A 2. Pharmacokinetics and immune response. *J. Natl. Cancer Inst. 80:937.*
13. **Byers, V. S., and R. W. Baldwin.** 1988. Therapeutic strategies with monoclonal antibodies and immunoconjugates. *Immunology 65:239.*
14. **Carson, D. A., and B. D. Freimark.** 1986. Human lymphocyte hybridomas and monoclonal antibodies. *Adv. Immunol. 38:275.*
15. **Morrison, S. L.** 1985. Transfectomas provide novel chimeric antibodies. *Science 229:1202.*
16. **Morrison, S. L., M. J. Johnson, L. A. Herzenberg, and V. T. Oi.** 1984. Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. *Proc. Natl. Acad. Sci. USA 81:6851.*
17. **Reichmann, L., M. Clark, H. Waldmann, and G. Winter.** 1988. Reshaping human antibodies for therapy. *Nature 332:323.*
18. **Tan, L. K., and S. L. Morrison.** 1988. Antibody structure and anti-

body engineering. *Adv. Drug Deliv. Rev. 2:129.*

19. **Shaw, D. R., G. Harrison, L. K. Sun, C. W. Shearman, S. McKinney, P. E. Dadonna, and A. F. LoBuglio.** 1988. Human lymphocyte and monocyte lysis of tumor cells mediated by a mouse/human chimeric monoclonal antibody. *J. Biol. Response Modifiers 7:204.*
20. **Brown, B. A., G. L. Davis, J. Saltzberger-Muller, P. Simon, M.-K. Ho, P. S. Shaw, B. A. Stone, H. Sands, and G. P. Moore.** 1987. Tumor specific genetically engineered murine/human chimeric monoclonal antibody. *Cancer Res. 47:3577.*
21. **Nishimura, Y., M. Yokoyama, K. Araki, R. Ueda, A. Kudo, and T. Watanabe.** 1987. Recombinant human-mouse chimeric monoclonal antibody specific for common acute lymphocytic leukemia antigen. *Cancer Res. 47:999.*
22. **Liu, A. Y., R. Robinson, K. E. Hellstrom, E. D. Murray, C. P. Chang, and I. Hellstrom.** Chimeric mouse-human IgG1 antibody that can mediate lysis of cancer cells. *Proc. Natl. Acad. Sci. USA 84:3439.*
23. **Li, Y.-W., D. K. Lawrie, P. Thammana, G. P. Moore, and C. W. Shearman.** 1990. Construction, expression and characterization of a murine/human chimeric antibody with specificity for hepatitis B surface antigen. *Mol. Immunol. 27:303.*
24. **Shearman, C. W., E. J. Kanzy, D. K. Lawrie, Y.-W. Li, P. Thammana, G. P. Moore, and R. Kurrle.** 1991. Construction, expression and biological activity of murine/human chimeric antibodies with specificity for the human alpha/beta T cell receptor. *J. Immunol. 146:928.*
25. **Shlom, J.** 1988. Monoclonal antibodies in cancer therapy: the present and the future. *Biopharmacology 1:44.*
26. **Steplewski, Z., L. K. Sun, C. W. Shearman, P. Dadonna, and H. Koprowski.** 1988. Biological activity of human-mouse IgG1, IgG2, IgG3 and IgG4 chimeric monoclonal antibodies with antitumor specificity. *Proc. Natl. Acad. Sci. USA 85:4852.*
27. **LoBuglio, A. F., R. H. Wheeler, J. Trang, A. Haynes, K. Rogers, E. B. Harvey, L. Sun, and M. B. Khazeli.** 1989. Mouse/human chimeric monoclonal antibody in man: kinetics and immune response. *Proc. Natl. Acad. Sci. USA 86:4220.*
28. **Jones, P. T., P. H. Dear, J. Foote, M. S. Neuberger, and G. Winter.** 1986. Replacing the complementarity determining regions in a human antibody with those from a mouse. *Nature 321:522.*
29. **Verhoeyen, M., C. Milstein, and G. Winter.** 1988. Reshaping human antibodies: grafting an antilysozyme activity. *Science 239:1534.*
30. **Queen, C., W. P. Schneider, H. E. Selick, P. W. Payne, N. F. Landolfi, J. F. Duncan, N. M. Avdalovic, M. Levitt, R. P. Junghans, and T. A. Waldmann.** 1989. A humanized antibody that binds to the interleukin 2 receptor. *Proc. Natl. Acad. Sci. USA 86:10029.*
31. **Chen, E. Y., and P. H. Seaberg.** 1985. Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. *DNA 4:165.*
32. **Kurrle, R., W. Seyfert, A. Trautwein, and F. R. Seiler.** 1986. T cell activation by CD3 antibodies. In *Leukocyte Typing II.* E. L. E. Reinherz, ed. Springer-Verlag, New York. p. 137.
33. **Schrezenmeier, H., R. Kurrle, H. Wagner, and B. Fleischer.** 1985. Activation of human T lymphocytes III. Triggering of bystander cytotoxicity in cytotoxic T cell clones by antibodies against the T3 antigen or by a calcium ionophore. *Eur. J. Immunol. 15:1019.*
34. **Kabat, E. A., T. T. Wu, M. Reid-Miller, N. M. Perry, and K. S. Gottesman.** 1987. In *Sequences of Proteins of Immunological Interest.* United States Department of Health and Human Services, United States Government Printing Office, Washington, DC.
35. **Chothia, C., A. M. Lesk, M. Levitt, A. G. Amit, R. A. Mariuzza, S. E. V. Phillips, and R. J. Poljak.** 1986. The predicted structure of immunoglobulin D1.3 and its comparison with the crystal structure. *Science 233:755.*
36. **Panka, D. J., M. Mudgett-Hunter, D. R. Parks, L. L. Peterson, L. A. Herzenberg, E. Haber, and M. N. Margolies.** 1988. Variable region framework differences results in decreased or increased affinity of variant anti-digoxin antibodies. *Proc. Natl. Acad. Sci. USA 85:3080.*
37. **Rudikoff, S., A. M. Giusti, W. D. Cook, and M. D. Scharff.** 1982. Single amino acid substitution altering antigen binding specificity. *Proc. Natl. Acad. Sci. USA 79:1979.*
38. **Tonegawa, S.** 1983. Somatic generation of antibody diversity. *Nature 302:575.*
39. **Soebandrio, A., T. Azuma, Y. Hamada, N. Sakato, and H. Fujio.** 1987. Specificity change of antibody to (4-hydroxy-3-nitrophenyl) acetyl haptens by somatic hypermutation. *J. Biochem. 102:1337.*
40. **Gearhart, P., N. D. Johnson, R. Douglas, and L. Hood.** 1981. IgG antibodies to phosphorylcholine exhibit more diversity than their IgM counterparts. *Nature 291:29.*
41. **Bruggermann, M., H. J. Miller, C. Burger, and K. Rajewski.** 1986. Idiotypic selection of an antibody mutant with changed hapten binding specificity resulting from a point mutation in position 50 of the heavy chain. *EMBO J. 5:1561.*

0022-1767/91/1463-0928$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 146, 928-935, No. 3, February 1, 1991
Printed in U.S.A.

CONSTRUCTION, EXPRESSION, AND BIOLOGIC ACTIVITY OF MURINE/HUMAN CHIMERIC ANTIBODIES WITH SPECIFICITY FOR THE HUMAN α/β T CELL RECEPTOR

CLYDE W. SHEARMAN,[1]* E. J. KANZY,† DAWSON K. LAWRIE,* YI-WAN LI,[2]* PALLAIAH THAMMANA,[3]* GORDON P. MOORE,[4]* AND ROLAND KURRLE†

*From *Genzyme Corporation, Framingham, MA 01701; and †Behringwerke Aktiengesellschaft, D-3550 Marburg, Federal Republic of Germany*

Murine/human chimeric antibodies with specificity for the human TCR-α/β have been produced by genetic engineering. The L and H chain V region exons encoding the murine mAb BMA 031 were isolated and inserted into mammalian expression vectors containing the human κ and γ1 or γ4 C region exons. The chimeric genes were transfected into murine Sp2/0 hybridoma cells by electroporation and transfectomas secreting chimeric antibody were isolated. Secretion levels ranged from 1 to 7 pg/cell/24 h. The chimeric antibodies bound specifically to T cells and competed effectively with the parental murine mAb for binding to these sites. The ability to promote antibody-dependent cell-mediated cytolysis was significantly enhanced in the chimeric antibodies as compared with murine BMA 031. C-dependent cytolysis, however, was not detectable with any of the antibodies. Chimeric BMA 031 is a clinically relevant, genetically engineered antibody with potential uses in transplantation, graft-vs-host disease, autoimmune diseases and other T cell-related disorders.

mAb directed against human T cells have been used extensively to analyze mechanisms of T cell activation and human T cell imbalances in various diseases (1-3). Furthermore, some of these antibodies seem to be powerful therapeutic agents in the treatment of leukemias, renal allograft rejection, acute graft-vs-host disease and autoimmune diseases (4-6).

Despite the fact that mAb directed against T cells have been applied to patients for almost 10 yr (7, 8), their in vivo mechanisms of action are not yet completely understood. The particular Ag recognized by the mAb seems to play a crucial role in its therapeutic effectiveness. Also, in addition to the specificity of the mAb, Fc-mediated reactions influence in vitro as well as in vivo effects. Therefore, the selection of an appropriate antibody isotype may be as important for therapeutic efficacy as the selection of an appropriate specificity.

The human TCR-α/β is expressed on all mature T lymphocytes and is thought to recognize Ag in the context of MHC molecules. This heterodimer is noncovalently linked to the multi-chain CD3-Ag complex, which is strongly involved in signal transduction and amplification after binding of Ag to the TCR-α/β (9). Under in vitro experimental conditions, T cells can be activated by providing trigger signals via the CD3-Ag complex. Binding of anti-CD3 mAb, like OKT3 or BMA 030, followed by physiologic or artificial cross-linking of the mAb usually results in polyclonal T cell activation (4). Under physiologic conditions, binding of mAb to the CD3-Ag complex results in a so-called "signal one" of T cell activation. A second activation signal is derived from accessory cells, provided the Fc part of the Ig molecule is able to interact with FcR on the accessory cells (10). Only under conditions in which both signals are available is T cell activation complete, resulting in lymphocyte proliferation (11, 12).

Until recently, all mAb directed to epitopes of the CD3/TCR complex were thought to deliver the first signal of T cell activation. However, in vitro studies with the anti-human TCR-α/β mAb BMA 031 have shown that this antibody delivers different signals of T cell activation than anti-CD3 mAb even of the same isotype (13). These differences are most likely caused by the specificities of these mAb. In addition, because BMA 031 is of the murine IgG2b isotype and thus does not interact with the majority of human FcR (10), binding of BMA 031 to human TCR usually does not result in induction of secondary signals from accessory cells. This leads to incomplete T cell activation, weak T cell proliferation, and the release of very small amounts of cytokines. These properties seem to be essential with respect to clinical application. In clinical trials, where BMA 031 is used for prophylaxis and therapy of both graft rejections in organ transplantation and graft-vs-host disease after bone marrow transplantation, this antibody is very well tolerated (14, 15).

Most mAb used in human therapy are of murine origin and may have limited utility because they usually elicit an immune response in patients (16, 17). Such a response reduces therapeutic efficacy and also may cause undesired clinical side effects. Human mAb are expected to circumvent this problem but they are difficult to produce

Received for publication June 28, 1990.
Accepted for publication October 30, 1990.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and reprint requests to C. W. Shearman, Genzyme Corporation, One Mountain Road, Framingham, MA 01701.
[2] Present address: Chinese Academy of Medical Sciences, Beijing, China.
[3] Present address: E.I. DuPont de Nemours and Co., Glasgow Site, Building 700, Newark, DE 19717.
[4] Present address: Department of Molecular Genetics, Smith Kline Beecham, 709 Swedeland Road, King of Prussia, PA 19406.

and often have low affinity. Moreover, human hybridomas are usually unstable and secrete Ig at low levels (18, 19). Chimeric antibodies consisting of murine V regions and human C regions represent a compromise. Conceptually, a chimeric antibody would retain the affinity and specificity of the parental murine mAb, and would eliminate the patient immune response to the murine C regions. Also, transfectomas producing chimeric antibodies appear to be stable and secrete antibody at levels sufficient for commercial application (20, 21). A number of chimeric antibodies have been produced recently (22–24) and some are entering clinical trials (25). Here, we report the production of chimeric antibodies with the affinity and specificity of BMA 031, a murine mAb specific to the human TCR-α/β (4). These antibodies were used to study the Fc-mediated components of T cell activation. Moreover, chimeric BMA 031 antibodies also display enhanced ADCC[5] activity and may have advantages in vivo, especially in situations in which elimination of T cells is required. Chimeric BMA 031 may, therefore, have efficacy for both immunoregulation and treatment of T cell-related disorders, like T cell leukemias.

MATERIALS AND METHODS

Cell culture. The BMA 031, P3x63.Ag8, and Sp2/0-Ag14 hybridomas were cultured in DMEM supplemented with 10% FCS, 10 mM HEPES (pH, 7.3), 2 mM L-glutamine, 10 mM nonessential amino acids (GIBCO, Grand Island, NY), and 10 mM pyruvate. Transfectomas were grown in the above medium containing 1 μg/ml mycophenolic acid, 50 μg/ml xanthine, and 500 μg/ml Geneticin (GIBCO). For antibody production, hybridoma or transfectoma cells were cultured in serum-free Iscove's medium. HPB-ALL and CEM cells were also cultured in serum-free Iscove's medium. All lines were maintained at 37°C in 5 to 7% CO_2.

Nucleotide sequencing. The nucleotide sequence of both the H and L chain V regions of BMA 031 mRNA was determined by the primer extension method (27) using avian myeloblastosis virus reverse transcriptase. Total RNA was extracted from BMA 031 cells with guanidinium thiocyanate (28) and poly A^+ mRNA was isolated by oligo (dT) cellulose chromatography (29). Universal primers, corresponding to the C regions of H and κL chains, were used in the initial sequencing (30). DNA sequencing of the cloned VH and VL regions was performed directly on pUC subclones by using universal forward and reverse primers (31). Additional primers were synthesized on an Applied Biosystems (Foster City, CA) model 380A DNA synthesizer to complete the sequencing.

Isolation of BMA 031 V_H and V_L regions from a genomic library. A genomic DNA library from the BMA 031 cell line was constructed in the λ phage vector EMBL-3. High m.w. DNA was isolated, partially digested with restriction endonuclease Sau3A, and fractionated by agarose gel electrophoresis (32). DNA fragments between 9 and 23 kb were eluted onto a glass fiber filter, extracted, and ethanol precipitated. The DNA was then ligated with λ EMBL-3 that had been digested with *Bam*HI and dephosphorylated. Recombinant λ phage were packaged with GIGAPACK GOLD extract (Stratagene, San Diego, CA) and plated at a density of 4.4×10^5/150 mm diameter petri dish. Duplicate filter lifts were prepared by using nitrocellulose filters (Schleicher and Schuell, Keene, NH). Filters were hybridized with random oligodeoxynucleotide-labeled probes (33). The probe used for identifying the V_H region was the 1.3 kb *Hin*dIII/*Pst*I DNA fragment (HPH) containing murine J_{H4} and a portion of the intron between the V and C region of the H chain. The probe used for detecting the V_L region was the 1.1-kb *Pst*I/*Hin*dIII DNA fragment (HPL) derived from the murine L chain intron (see Fig. 1). Putative positive clones were isolated and purified by up to four rounds of rescreening.

Transfection of DNA into mouse cells by electroporation. DNA was introduced into murine hybridoma Sp2/0-Ag14 cells by electroporation. 1 to 2×10^7 actively growing Sp2/0-Ag14 cells were washed and resuspended in 1.0 ml of sterile PBS. Fifteen micrograms of each chimeric, Igκ and IgG1 (or IgG4), plasmid (linearized with *Bam*HI) were added to the cell suspension. The DNA/cells were transferred to a precooled shocking cuvette, incubated on ice at least 5 min and then a 0.5 kv/cm electric pulse was delivered for 10 ms (Transfector 300, BTX, San Diego, CA). After shocking, the DNA/cell mixture was returned to ice for 10 min and then diluted in 40 ml of supplemented DMEM and incubated at room temperature for 10 min. Finally, the cells were transferred to a 37°C incubator with 7% CO_2 for 48 h before plating in selective medium, containing 1 μg/ml mycophenolic acid, 50 μg/ml xanthine and 1 mg/ml Geneticin. Cells were plated in 96-well plates at 3×10^4 cells/well.

T cell proliferation assay. Ficoll-separated PBL were cultured in serum-free Iscove's medium at 5×10^4 cells/well in 96-well U-shape microtiter plates in the presence of various concentrations of anti-CD3 or BMA 031 antibodies (0 to 10 μg/ml). Cells were cultured in quadruplicate for 3 or 6 days at 37°C. [^{14}C]TdR (75 nCi/well; New England Nuclear, Boston, MA) was added during the last 16 h of culture. Cells were collected by an automated cell harvester and analyzed by an automatic filter counting system (Innotech Trace 96, Innotech, Trumbull, CT).

Cytofluorometric assays for specificity and affinity. Heparinized HPB cells from healthy volunteers were incubated at 4°C for 30 min in the presence of BMA 031 culture supernatants, purified Ig (BMA 031, BMA 031-G1, BMA 031-G4), or with FITC-conjugated BMA 031 antibodies (direct immunofluorescence assay). In indirect assays, FITC-conjugated rabbit anti-mouse Ig F(ab')$_2$ or anti-human Ig F(ab')$_2$ antibodies were used as second-step reagents. In several experiments, cells were preincubated with polyclonal human Ig (Beriglobin, Behringwerke, Germany) to reduce nonspecific binding of mAb to FcR. All mAb were used at concentrations twice that required for Ag saturation. To analyze the relative affinities of murine and chimeric BMA 031 antibodies, competitive immunofluorescence assays were carried out. PBMC were separated by Ficoll-Hypaque density gradient centrifugation and incubated with mAb at various concentrations (0.01 to 10 μg/ml) for 30 min. After removing unbound antibodies by two washing steps, cells were incubated with 10 μg/ml of FITC-conjugated BMA 031 antibodies for 30 min. Cells from all experiments were analyzed either on an Ortho (Raritan, NJ) Cytofluorograph 50H/2150 computer system or on a Becton Dickinson (Mountain View, CA) FACStar Plus as described elsewhere (26). The percentage of fluorescein-positive cells was calculated by modified Ortho or standard FACStar Plus software.

Cytotoxicity assays. To measure the cytolytic capacity of the BMA 031 antibody preparations, a 20-h ^{51}Cr-release assay was performed to measure ADCC and NK activity. ^{51}Cr-labeled HPB-ALL target cells were incubated with (ADCC) or without (NK activity) various concentrations of antibodies for 20 h in the presence of Ficoll-separated PBL (effector cells). TCR-α/β-negative CEM cells were used as control target cells. The antibodies were allowed to bind first to target cells (30 min) before the effector cells were added. The E:T ratio varied from 1:1 to 50:1. Cytolysis in the absence of antibodies was considered to be caused by NK activity. The percentage of specific lysis was calculated as described earlier (34). Spontaneous ^{51}Cr release in the absence of effector cells was always less than 5%. All samples were analyzed in triplicate. CDC was measured in a standard lymphocytotoxicity test as used routinely to measure AHLG-cytolysis titers (Pressimmune, Behringwerke, Germany) (35).

Preparation of F(ab')$_2$ molecules. BMA 031-G1 and BMA 031-G4 (1 mg/ml) were cleaved with pepsin (9000 FIP-U/g, Merck, Darmstadt, Germany) at an antibody to pepsin ratio of 20:1. BMA 031-G1 was digested in 100 mM sodium citrate (pH 3.5) at 37°C for 30 min. BMA 031-G4 was digested in 100 mM sodium citrate (pH 4.0) at 37°C for 2 h. Digestion was stopped by adjusting the pH to 7.5 with 2 M Tris-HCl. After concentration with a Centricon 30 (Amicon, Danvers, MA) IgG F(ab')$_2$ was separated from intact IgG and smaller fragments by size exclusion chromatography on Ultragel AcA34 (IBF Biotechnics, Savage, MD) by using 20 mM Tris-HCl (pH 7.5) and 400 mM NaCl. The F(ab')$_2$ fraction was pooled and passed over a protein A Sepharose CL4B (Pharmacia, Piscataway, NJ) column in 50 mM Tris-HCl (pH 8.6) and 150 mM NaCl. Unbound protein was concentrated by ultrafiltration and dialyzed against 10 mM sodium phosphate (pH 7.2) and 140 mM NaCl. Purity of the IgG F(ab')$_2$ preparations was checked by SDS-PAGE and an ELISA by using mAb specific for the Fc portion of human IgG1 or IgG4.

RESULTS

Restriction analysis and mRNA sequencing of the BMA 031 V regions. Genomic DNA from BMA 031 and its fusion partner, P3x63.Ag8, was digested with various restriction endonucleases and analyzed by Southern blotting to identify the functionally rearranged V regions.

[5] Abbreviations used in this paper: ADCC, antibody-dependent cell-mediated cytolysis; ALL, acute lymphocytic leukemia; CDC, C-dependent cytolysis; CDR, complementarity-determining region; HPB, human peripheral blood; V_H, variable H chain; V_L, variable L chain.

This analysis showed that the H chain V region was contained in a 5.6-kb *Eco*RI fragment and that the L chain V region was in a 3.0-kb *Hin*dIII fragment. Partial restriction enzyme maps (later confirmed and extended by DNA sequencing of the genomic clones) are shown in Figure 1. In order to synthesize BMA031-specific oligomer probes for screening genomic clones, and to confirm that the correct genes had been cloned, the V_H and V_L regions of BMA 031 poly A^+ mRNA was sequenced. Nucleotide sequences of approximately 200 bases of the V regions of H and L chains were obtained from the initial sequencing reaction. Additional primers were synthesized based on this sequence in order to obtain the complete sequence. Probes corresponding to CDR3 of both the L and H chains were synthesized and used to screen genomic libraries (see Fig. 2).

Isolation of VH and VL regions of BMA 031. A genomic library of BMA 031 DNA was prepared in λ-phage EMBL-3. The library, 2×10^6 phage, was screened initially with the fragment probes HPH and HPL. The BMA 031 V region-specific oligomer probes were used in subsequent rounds of rescreening to isolate pure plaques. Four H chain clones and seven L chain clones were identified which hybridized strongly to both the fragment and BMA 031-specific oligomer probes. Southern analysis showed that the H chain clones contained the expected 5.6-kb *Eco*RI fragment and that the L chain clones contained the 3.0 kb *Hin*dIII fragment (see Fig. 1). The 3.0-kb *Hin*dIII V_L fragment and the 5.6-kb *Eco*RI V_H fragment were subcloned into pUC19.

DNA sequencing of the V_H and V_L regions of BMA 031. The 1.1-kb *Hin*dIII V_H fragment and the 1.4 kb *Eco*RI/*Hin*cII V_L fragment were subcloned into pUC19 and sequenced directly by the dideoxy method. The coding sequences are shown in Figure 2. The positions of the signal sequences, CDR, and oligomer probes are indicated. Analysis of the sequences indicates that BMA 031 V_H is derived from the JH_3 minigene and is a member of subgroup IIB (36). It was also found that the D segment in BMA 031 is derived from DSP 2.2. The sequencing also demonstrated that BMA 031 V_L is derived from the JK_5 minigene and belongs to κ subgroup VI.

Construction and expression of chimeric BMA 031 H and L chain genes. The 5.6-kb *Eco*RI BMA 031 VH fragment was cloned into the *Eco*RI site of mammalian expression vectors which contain either the human gamma-1 or gamma-4 C region and the *gpt* gene (Fig. 3*A*). The 3.0-kb *Hin*dIII BMA 031 VL fragment was cloned into the *Hin*dIII site of a similar vector that contains the human κ C region and the *neo* gene (Fig. 3*B*). The two plasmids were co-transfected into Sp2/0-Ag14, a non-Ig-producing murine hybridoma, by electroporation. Transfection efficiency was approximately 1×10^{-5}. After 2 wk of drug selection, culture supernatants were assayed for the presence of chimeric antibodies produced by the transfectomas.

Analysis of chimeric BMA 031 transfectomas. Culture supernatants from drug-resistant cells were assayed for the presence of murine/human chimeric antibody by ELISA. The antibody used to coat the microtiter plates was goat anti-human IgG (Fc specific) and goat anti-human κ antibody conjugated to horseradish peroxidase was used to detect Ag-antibody complexes. The 10 highest producing transfectomas from each transfection (γ1 and γ4) were assayed for antibody production daily for 1 wk. Chimeric antibody was produced at a rate of 1.2 to 7.0 pg/cell/24 h (data not shown). None of the chimeric antibodies reacted with goat anti-mouse antibody. The best clone with respect to secretion level and growth characteristics from each transfection was subcloned and expanded for further study. The clones are referred to as BMA 031-G1 (human IgG1 chimeric) and BMA 031-

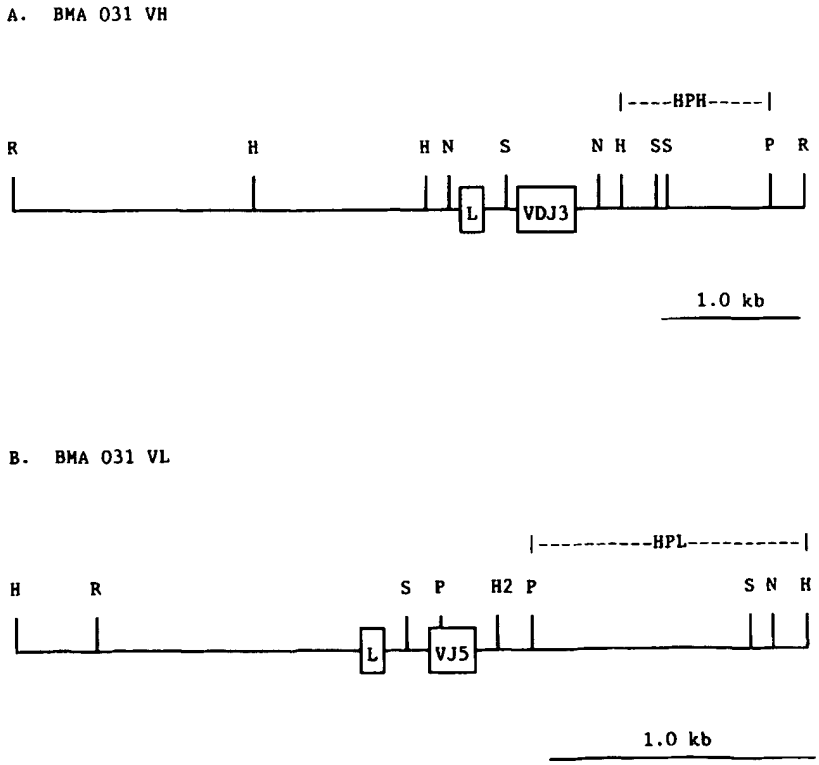


Figure 1. Partial restriction enzyme maps of BMA 031 V regions. *A*, the 5.6-kb *Eco*RI fragment containing the VDJ_3 exon; *B*, the 3.0 kb *Hin*dIII fragment containing the VJ_5 exon. *H*, *Hin*dIII; *H2*, *Hin*cII; *N*, *Nsi*I; *P*, *Pst*I; *R*, *Eco*RI; *S*, *Sau*I.

A. BMA 031 VH

```
CTAACCATGGAATGGAGTTGGATATTTCTCTTTCTCCTGTCAGGAACTGCAGGTAAGGGG
     M  E  W  S  W  I  F  L  F  L  L  S  G  T  A
  |--------------Signal Sequence-----------------

CTCACCAGTTCAGTCAAATCTGAAGTGGAGACACAGGACCTGAGGTGACAATGACATCTA

CTCTGACATTCTCTCCTCAGGTGTCCACTCTGAGGTCCAGCTGCAGCAGTCTGGACCTGA
                  G  V  H  S  E  V  Q  L  Q  Q  S  G  P  E
                  ----------| 1

GCTGGTAAAGCCTGGGGCTTCAGTGAAGATGTCCTGCAAGGCTTCTGGATATAAATTCAC
 L  V  K  P  G  A  S  V  K  M  S  C  K  A  S  G  Y  K  F  T

TAGCTATGTTATGCACTGGGTGAAGCAGAAGCCTGGGCAGGGCCTTGAGTGGATTGGATA
 S  Y  V  M  H  W  V  K  Q  K  P  G  Q  G  L  E  W  I  G  Y
|----CDR 1----|                                          |--

TATTAATCCTTACAATGATGTTACTAAGTACAATGAGAAGTTCAAAGGCAAGGCCACACT
 I  N  P  Y  N  D  V  T  K  Y  N  E  K  F  K  G  K  A  T  L
--------------------CDR 2----------------------|

GACTTCAGACAAATCCTCCAGTACAGCCTACATGGAGCTCAGCAGCCTGACCTCTGAGGA
 T  S  D  K  S  S  S  T  A  Y  M  E  L  S  S  L  T  S  E  D

                              *-----VH Probe-----*
CTCTGCGGTCCATTACTGTGCAAGAGGGAGCTACTATGATTACGACGGGTTTGTTTACTG
 S  A  V  H  Y  C  A  R  G  S  Y  Y  D  Y  D  G  F  V  Y  W
                        |------------CDR 3--------------|

GGGCCAAGGGACTCTGGTCACTGTCTCTGCAGGTGAGTCCTAACTTCTCCCATTCTAAAT
 G  Q  G  T  L  V  T  V  S  A
          JH3
```

B. BMA 031 VL

```
AAAATGGATTTTCAAGTGCAGATTTTCAGCTTCCTGCTAATCAGTGCCTCAGGTAACAGA
   M  D  F  Q  V  Q  I  F  S  F  L  L  I  S  A  S
  |----------------Signal Sequence-----------------

GGGCAGGGAATTTGAGATCAGAATACAACCAAAATTATTTTCCCTGGGGAATTTGTGTCC

AAAATACAGTTTTTTCTTTTTCTTTTATCTAAATGTTGGGTGGTATAAAATTATTTTTTA

TCTCTATTTCTACTAATCCCTCTCTCTTTTTTGCTTTTTTCTAGTCATAATATCCAGAGG
                                                V  I  I  S  R  G
                                                ----------------|

ACAAATTGTTCTCACCCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAAGGTCAC
 Q  I  V  L  T  Q  S  P  A  I  M  S  A  S  P  G  E  K  V  T
 1

CATGACCTGCAGTGCCACCTCAAGTGTAAGTTACATGCACTGGTACCAGCAGAAGTCAGG
 M  T  C  S  A  T  S  S  V  S  Y  M  H  W  Y  Q  Q  K  S  G
          |------------CDR 1-----------|

CACCTCCCCCAAAAGATGGATTTATGACACATCCAAACTGGCTTCTGGAGTCCCTGCTCG
 T  S  P  K  R  W  I  Y  D  T  S  K  L  A  S  G  V  P  A  R
                        |-------CDR 2-------|

CTTCAGTGGCAGTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCAGCATGGAGGCTGA
 F  S  G  S  G  S  G  T  S  Y  S  L  T  I  S  S  M  E  A  E

                                  *-----VL Probe-----*
AGATGCTGCCACTTATTACTGCCAGCAGTGGAGTAGTAACCCGCTCACGTTCGGTGCTGG
 D  A  A  T  Y  Y  C  Q  Q  W  S  S  N  P  L  T  F  G  A  G
                        |----------CDR 3----------|     JK5

GACCAAGCTGGAGCTGAAACGTAAGTACACTTTTCTCATCTTTTTTTATGTGTAAGACAC
 T  K  L  E  L  K
```

Figure 2. DNA sequences encoding the V exons of BMA 031. *A*, the BMA 031 VH coding sequence; *B*, the BMA 031 VL coding sequence, each showing the signal sequence, start of the mature protein, the CDR, the J region and the V-specific probe used to isolate the V regions.

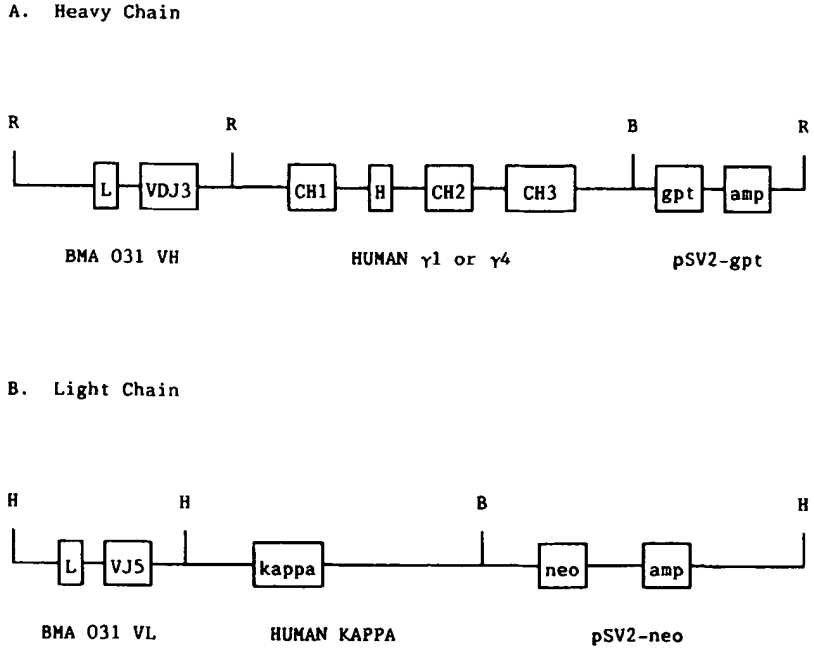


Figure 3. Expression vectors for chimeric Ig genes. *A*, the H chain expression vectors containing the BMA 031 VH region, the human γ1 or γ4 C region and the guanine phosphoribosyl transferase gene for selection. *B*, the L chain expression vector containing the BMA 031 VL region, the human κ C region, and the neomycin resistance gene for selection.

G4 (human IgG4 chimeric).

Purification and characterization of chimeric BMA 031 antibodies. The antibodies secreted by BMA 031-G1 and BMA 031-G4 were tested to ensure that they were indeed BMA 031 mouse/human chimeric antibodies. A series of ELISA assays showed that the antibodies contain human κ and γ C regions. Moreover, the antibodies did not react with antibodies directed against murine κ or γ C regions. Isotyping reagents also confirmed that the chimeric antibodies were of the IgG1 and IgG4 isotypes (data not shown).

The BMA 031-G1 and BMA 031-G4 transfectomas were expanded and supernatant was collected from each line. Saturated cultures accumulate antibody to at least 35 μg/ml for BMA 031-G1 and 15 μg/ml for BMA 031-G4. The chimeric antibodies were partially purified by protein A Sepharose column chromatography by using a linear pH gradient for elution (100 mM sodium citrate, pH 3 to 7).

Analysis of the antibodies by reducing and nonreducing SDS-PAGE showed a high degree of purity; although both chimeric antibodies showed some aggregation (data not shown). Also, BMA 031-G4 appeared to be more sensitive to reduction than BMA 031-G1, forming half-molecules in the presence of trace amounts of 2-ME.

Specificity and affinity of BMA 031-G1 and BMA 031-G4. The murine BMA 031 antibody is known to react with the α/β-chains of the human TCR (37). Because the correct specificity of an antibody is essential for all functional analyses, it was important to establish that both chimeric antibodies have an identical specificity as murine BMA 031. In indirect immunofluorescence assays, crude culture supernatants or purified protein of BMA 031-G1 and BMA 031-G4 bind to PBL to the same extent as murine BMA 031. In addition, when using either purified T cells (E^+ cells) or directly labeled antibodies, both chimeric antibodies possess an identical specificity as compared with murine BMA 031 (data not shown).

The relative affinities of the BMA 031 antibodies were compared by competitive immunofluorescence assays. For this purpose, human PBL were preincubated with various concentrations of either BMA 031, BMA 031-G1, or BMA 031-G4. In a second incubation step, cells were stained with BMA 031-FITC and analyzed in a cell sorter. The data shown in Figure 4*A* indicate that murine and chimeric BMA 031 antibodies block the binding of BMA 031-FITC in the same dose-dependent manner. If either BMA 031-G1-FITC or BMA 031-G4-FITC was used as second-step reagent, identical results were obtained (Fig. 4, *B* and *C*). These data clearly demonstrate that murine and chimeric BMA 031 antibodies have very similar relative affinities.

T cell activation by anti-CD3 and BMA 031 antibodies. Previous studies with BMA 031 and anti-CD3 antibodies have shown differences in their abilities to activate T cells because of differences in both the Fc and Ag specificity. It is of interest, therefore, to analyze T cell activation by chimeric BMA 031 mAb, which have both the specificity of BMA 031 and the capacity to interact with human FcR. In Figure 5, data of a representative experiment are shown. In a 3-day proliferation assay (Fig. 5*A*), BMA 030 (anti-CD3) induced a typical bell-shaped curve. BMA 031-G1 and BMA 031-G4 need higher antibody concentrations, as compared with BMA 030, for T cell stimulation, but are much more mitogenic than BMA 031. Because the BMA 031 antibodies differ only in the C region of the molecule, these differences in T cell activation must be attributed to Fc-mediated functions. Interestingly, with the BMA 031 chimeric antibodies, no high dose suppression effects were seen even at concentrations of up to 10 μg/ml. In principal, similar results should be obtained in a 6-day proliferation assay. However, BMA 031 triggers a much stronger proliferation at 6 days than at 3 days (Fig. 5*B*). Again, the chimeric antibodies stimulate T cells to a greater extent than BMA 031 and without high dose suppression. These data suggest that BMA 031 triggers T cell proliferation in a more Fc-independent fashion, whereas the chimeric antibodies are able to provide an additional stimulatory signal derived from accessory cells.

To address this hypothesis, we carried out proliferation experiments with purified T cells as well as with $F(ab')_2$ fragments of mAb. The data of two representative experiments, shown in Table I, demonstrate that under experimental conditions in which anti-CD3 mAb BMA 030 is unable to stimulate T cell proliferation caused by the inability of Fc-mediated cross-linking (absence of accessory cells in the CD7 population or use of $F(ab')_2$ fragments), the stimulatory capacity of BMA 031, BMA 031-G1, or BMA 031-G4 is not impaired. These data strongly support the idea that, besides Fc-mediated reactions, the specificity of BMA 031 is instrumental for the differences in T cell activation as compared with anti-CD3 mAb.

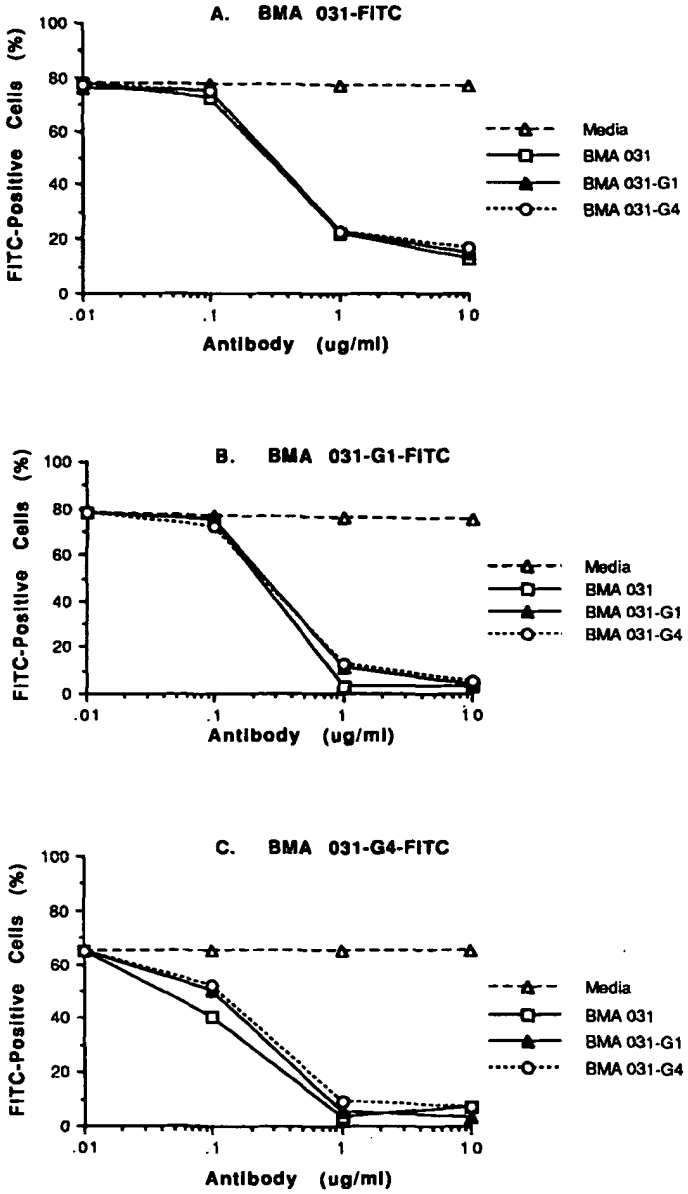


Figure 4. Relative affinities of BMA 031 antibodies. Competitive immunofluorescence assays with the BMA 031 antibodies, HPB mononuclear cells, and FITC-BMA 031 antibodies (10 μg/ml) were performed as outlined under *Materials and Methods*. *A*, BMA 031-FITC; *B*, BMA 031-G1-FITC; *C*, BMA 031-G4-FITC.

Cell cytotoxicity with BMA 031-G1 and BMA 031-G4. Murine mAb are usually not very effective in mediating cytolysis in either ADCC or CDC assays. In both cases, the Fc part of the molecule either does not allow optimal activation of the C cascade or provides an inappropriate interaction with human killer cells. Therefore, it was of interest to see if the human Fc part of the chimeric BMA 031 antibodies could alter the cytolytic capacity demon-

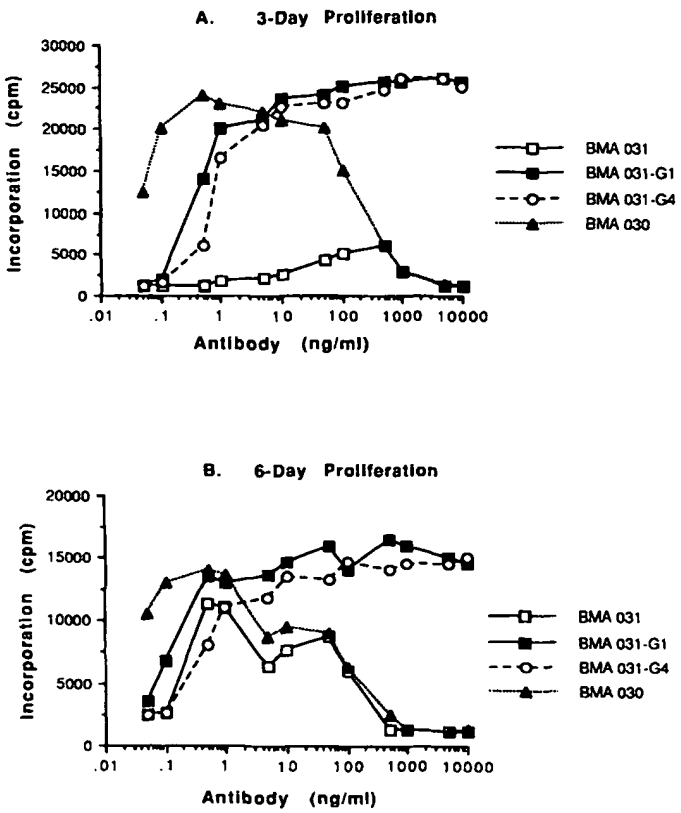


Figure 5. T cell activation by anti-CD3 and BMA 031 antibodies. *A*, a 3-day lymphocyte proliferation assay; *B*, a 6-day lymphocyte proliferation assay. Experimental conditions were as described under *Materials and Methods*. Medium and phytohemagglutinin controls (*A*) were 750 cpm and 24,500 cpm, respectively. Medium and splenic adherent cell controls (*B*) were 3,000 cpm and 12,500 cpm, respectively.

TABLE I

Induction of T cell proliferation

T Cell Activation by[a]	[^{14}C]TdR Incorporation (cpm[b])		
	Experiment 1	Experiment 2	
	n-PBL[c]	n-PBL	CD7$^+$ cells[d]
BMA 030	34,765 ± 5%	24,910 ± 10%	225 ± 30%
BMA 030-F(ab')$_2$	720 ± 3%	445 ± 8%	460 ± 28%
BMA 031	3,415 ± 1%	4,840 ± 15%	1,375 ± 30%
BMA 031-G1-F(ab')$_2$	2,635 ± 10%	3,485 ± 18%	4,540 ± 8%
BMA 031-G4-F(ab')$_2$	4,032 ± 4%	7,360 ± 15%	5,660 ± 30%
Medium	170 ± 15%	300 ± 20%	150 ± 30%

[a] Concentrations used were 1 ng/ml for BMA 030 and 10 μg/ml for the BMA 031 antibodies.
[b] Mean of four values in a 3-day proliferation assay.
[c] Ficoll-separated PBL.
[d] Isolated by sorting (FACstar plus).

strated by BMA 031.

In CDC assays, only murine BMA 031 was able to lyse peripheral blood T cells, provided a selected rabbit C batch was used. The titer is strongly dependent on the blood donor and C batch and can vary from 1/200 to 1/6000 (calculated for 1 mg protein/ml). When other C sources (guinea pig or human) or HPB-ALL target cells were used, no CDC could be detected with BMA 031. The chimeric antibodies were unable to mediate CDC under any of the conditions tested (data not shown).

Because the chimeric BMA 031 antibodies were able to interact efficiently with human FcR in the T cell proliferation assays, there was a strong possibility that they would have high ADCC titers as well. To evaluate the ADCC capacity of these mAb, we compared them with rabbit anti-GH-1 antiserum. This antiserum was the best out of eight rabbit anti-human T cell globulins in ADCC capacity. The data of a representative experiment are shown in Figure 6. Even at low E:T ratios (Fig. 6*A*) or extremely low antibody concentrations (Fig. 6, *B* and C), chimeric BMA 031 antibodies are highly potent in killing HPB-ALL cells. In contrast, murine BMA 031 is very poor at ADCC. Further experiments will be needed to identify, in more detail, the effector cells capable of interacting with BMA 031-G1 and BMA 031-G4.

DISCUSSION

We have joined the DNA segments encoding the murine VH and VL exons from the BMA031 mAb specific for the human TCR to the DNA segments encoding human γ-1 or γ-4 and κC regions. When the chimeric genes were introduced into non-Ig producing Sp2/0 cells, functional chimeric antibodies with an identical affinity and specificity as murine BMA 031 were assembled and secreted.

The biologic activities of the chimeric antibodies are clearly different from those of BMA 031. Both BMA 031-G1 and BMA 031-G4 stimulate human T cells to a much greater extent than BMA 031 and without high dose suppression. Murine anti-CD3 mAb of the IgG2a isotype, such as BMA 030 and OKT3, are known to be highly mitogenic for human T cells (26). For the induction of T cell proliferation via CD3 Ag, binding of mAb to the Ag must be followed by cross-linking of the Ig molecules. Under physiologic conditions, this is achieved by interaction of the Fc portion of the mAb with FcR on accessory cells. An additional signal derived from the accessory cells seems to be essential for complete T cell activation (26). The strength of anti-CD3-induced T cell proliferation is typically dose dependent, resulting in a bell-shaped dose response curve. As reported earlier (4), induction of T cell proliferation by BMA 031 is relatively weak and requires much higher antibody concentrations for a longer period of time for maximal stimulation. This low mitogenicity was ascribed predominantly to deficient interaction with human FcR (4, 10, 26). Recent studies, however, have suggested that anti-CD3 mAb and BMA 031 trigger T cells by different pathways (38). In our present studies, the chimeric BMA 031 antibodies have proved to be a powerful tool in discriminating between specificity and Fc-related contributions to signals involved in T cell activation. Our data strongly supports the hypothesis that the differences in T cell activation exhibited by BMA 031 as compared with anti-CD3 mAb is caused by differences in specificity as well as Fc-mediated reactions. Under physiologic conditions, in T cell activation mediated by chimeric BMA 031 antibodies, the activation signal derived from mAb binding to the TCR is superimposed on Fc-mediated trigger signals derived from accessory cells, whereas murine BMA 031 will deliver only the first signal. With chimeric BMA 031-F(ab')$_2$ fragments or accessory cell depleted T cells (CD7$^+$ cells), we could show, that in contrast to anti-CD3 mAb, BMA031 is able to trigger T cell proliferation in an Fc-independent way. Experiments with PBL, E$^+$ cells, and thymocytes, where accessory cell function was bypassed by co-stimulation with an anti-CD28 mAb, corroborate this Fc independence (39). In addition, some differences in T cell activation between anti-CD3 and BMA 031 mAb, such as triggering of Ca^{2+} influx, could only be explained by differences in specificity as well (13). Experiments are

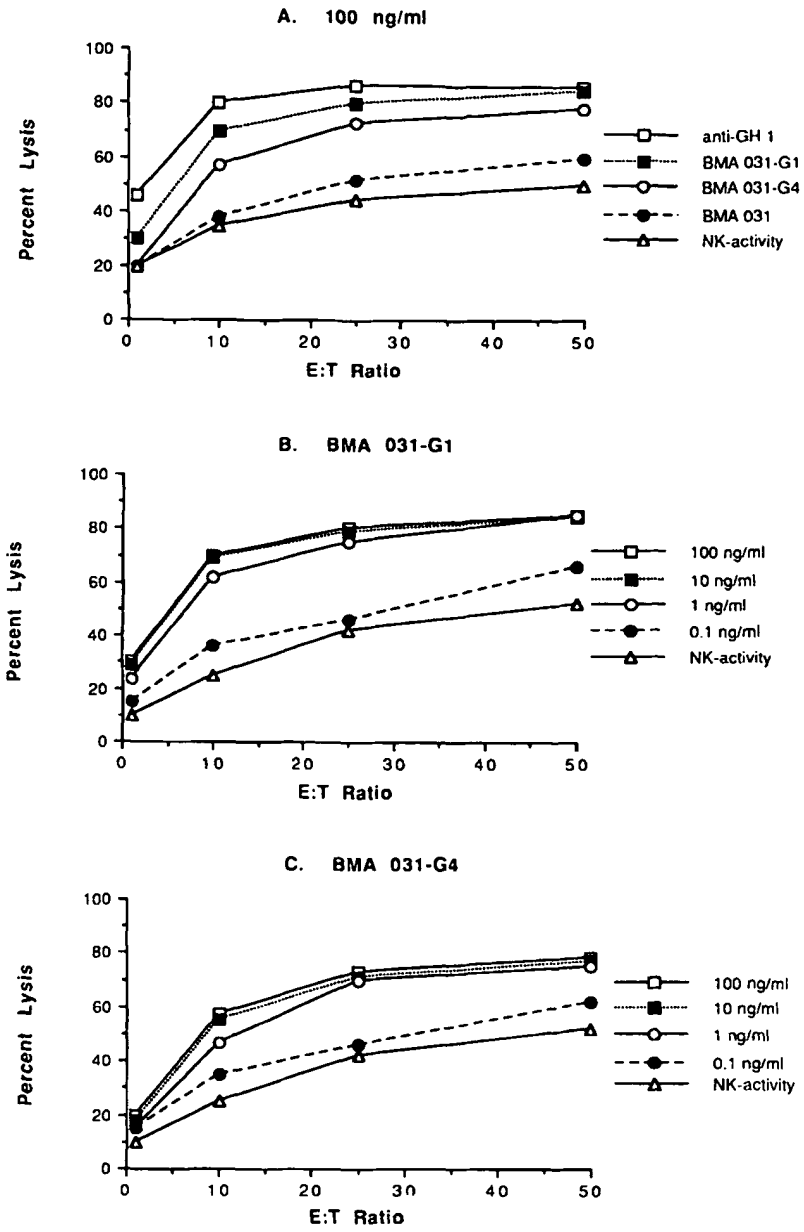


Figure 6. ADCC capacity of BMA 031 antibodies. The cytolytic capacity of the BMA 031 antibodies was determined in a 20-h ^{51}Cr release assay as described under *Materials and Methods*. *A*, lysis in the presence (ADCC) or absence (NK activity) of antibody (100 ng/ml); *B*, lysis at various concentrations of BMA 031-G1; *C*, lysis at various concentrations of BMA 031-G4.

in progress to identify the responding T cell population. Similar results have been shown with experiments in the murine system with anti-murine CD3 and anti-murine TCR-α/β mAb. On distinct T cell subpopulations, both mAb differ in the quality of signals transduced (9). Because incomplete T cell activation of selective T cell subpopulations may lead to anergy or apoptosis (40, 41), selective triggering of T cells by BMA 031 might be predominantly responsible for its immunoregulatory effects in vivo.

For therapeutic applications of mAb in T cell disorders, it may be advantageous to augment the cytolytic capacity of mAb. In particular, for treatment of T cell leukemias, a mAb capable of eliminating highly malignant tumor cells by means of the patients immune system might be superior to cytostatic drugs because only cells expressing a specific Ag are destroyed. Murine mAb are usually weak in cytolytic capacity and BMA 031 is no exception. Under various experimental conditions, only marginal cytolytic activity could be obtained with BMA 031. Compared with the strong ADCC activity achieved with selected polyclonal rabbit anti-T cell globulins (anti-GH1 antiserum), the ADCC activity obtained with different anti-leukocyte mAb, even of other specificities and isotypes, is weak. In contrast, both BMA 031-G1 and BMA 031-G4 are highly effective in cytolytic capacity. Even at low E:T ratios and extremely low antibody concentrations, the chimeric antibodies are highly potent in killing HPB-ALL cells.

The results presented suggest that chimeric BMA031 may have clinical utility in preventing transplant rejection, graft-vs-host disease, autoimmune diseases, and other T cell-related problems. The chimeric antibody retains the affinity and specificity of murine BMA 031 but contains human C regions, which should reduce or eliminate the patients immune response to the mouse C regions. Engineered BMA 031 antibodies with humanized V regions and human C regions have been produced recently (C. W. Shearman, D. P. Pollock, G. White, K. Hehir, G. P. Moore, E. J. Kanzy, and R. Kurrle, manuscript in preparation) and these should further reduce

the patients immune response during therapy. Moreover, effector functions associated with the human C regions are enhanced in vitro relative to murine BMA031 and may also be in vivo when the humanized antibodies are used for immunotherapy. We are now in a position to test the efficacy of humanized BMA031 in clinical trials.

Acknowledgments. We thank K. Hehir, J. Merrill, and G. White for the DNA sequencing and the synthesis of oligomers and K. Frank, W. Lang, and W. Seyfert-Brandt for expert technical assistance in immunologic assays. We are grateful to B. Herrera for typing the manuscript.

REFERENCES

1. **Reinherz, E. L., P. C. Kung, P. Goldstein, and S. F. Schlossman.** 1979. Separation of functional subsets of human T cells by a monoclonal antibody. *Proc. Natl. Acad. Sci. USA 76:4061.*
2. **Schlossman, S. F., and E. L. Reinhertz.** 1984. Human T cell subsets in health and disease. *Springer Semin. Immunopathol. 7:9.*
3. **van Dongen, J. J. M., H. J. Adriaansen, and H. Hooijkaas.** 1988. Immunophenotyping of leukaemias and non-Hodgkin's lymphomas: immunological markers and their CD codes. *Neth. J. Med. 33:298.*
4. **Kurrle, R., K. H. Enssle, and F. R. Seiler.** 1988. Monoclonal antibodies directed to leukocyte differentiation antigens for therapeutic use. *Behring Inst. Mitteilungen 82:154.*
5. **Ritz, J.** 1983. Use of monoclonal antibodies in autologous and allogeneic bone marrow transplantation. *Clin. Haematol. 12:813.*
6. **Russell, P. S., R. B. Colvin, and A. B. Cosimi.** 1984. Monoclonal antibodies for the diagnosis and treatment of transplant rejection. *Annu. Rev. Med. 35:63.*
7. **Nadler, L. M., P. Stashenko, R. Hardy, W. D. Kaplan, L. N. Button, D. W. Kufe, J. H. Antman, and S. F. Schlossman.** 1980. Serotherapy of a patient with a monoclonal antibody directed against a human lymphoma-associated antigen. *Cancer Res. 40:3147.*
8. **Miller, R. A., D. G. Maloney, J. McKillop, and R. Levy.** 1981. In vivo effects of murine hybridoma monoclonal antibody in a patient with T cell leukemia. *Blood 58:78.*
9. **Finkel, T. H., P. Marrack, J. W. Kappler, R. T. Kubo, and J. C. Cambier.** 1989. $\alpha\beta$ T cell receptor and CD3 transduce different signals in immature T cells: implications for selection and tolerance. *J. Immunol. 142:3006.*
10. **Smith, K. G. C., J. M. Austyn, G. Hariri, B. C. L. Beverley, and P. J. Morris.** 1986. T cell activation by anti-T3 antibodies: comparison of IgG1 and IgG2b switch variants and direct evidence for accessory function of macrophage Fc receptors. *Eur. J. Immunol. 16:478.*
11. **Martorell, J., R. Vilella, L. Borche, I. Rojo, and J. Vives.** 1987. A second signal for T cell mitogenesis provided by monoclonal antibodies CD45 (T200). *Eur. J. Immunol. 17:1447.*
12. **Schmidberger, R., T. Miethke, K. Heeg, and H. Wagner.** 1989. Primary activation of murine CD8 T cells via cross-linking of T3 cell surface structures: two signals regulate induction of interleukin 2 responsiveness. *Eur. J. Immunol. 18:277.*
13. **Wallace, D. L., E. A. Macintyre, D. C. Linch, and P. C. L. Beverley.** 1987. Analysis of the activation signals induced by CD3 antibodies and their role in T cell proliferation. In *Leukocyte Typing III.* A. J. E. McMichael, ed. Oxford University Press, Oxford, U.K., p. 167.
14. **Land, W.** 1989. Kidney transplantation: state-of-the-art. *Transplant. Proc. 21:1425.*
15. **Beelen, D. W., U. Graeven, G. Schulz, H. Grosse-Wilde, I. Doxiadis, U. W. Schaeffer, K. Guabeck, H. Sayer, and C. G. Schmidt.** 1988. Treatment of acute graft-versus-host disease after HLA-partially matched marrow transplantation with a monoclonal antibody (BMA 031) against the T cell receptor. First results of a phase I/II trial. *Onkologie 11:56.*
16. **Bach, J. F., and L. Chatenoud.** 1987. Immunologic monitoring of Orthoclone OKT3-treated patients: the problem of antimonoclonal immune response. *Transplant. Proc. 19:17.*
17. **Shawler, D. L., R. M. Bartholomew, L. M. Smith, and R. O. Dillman.** 1985. Human immune response to multiple injections of murine monoclonal IgG. *J. Immunol. 135:1530.*
18. **Carson, D. A., and B. D. Freimark.** 1986. Human lymphocyte hybridomas and monoclonal antibodies. *Adv. Immunol. 38:275.*
19. **Morrison, S. L.** 1985. Transfectomas provide novel chimeric antibodies. *Science 229:1202.*
20. **Dorai, H., and G. P. Moore.** 1987. The effect of dihydrofolate reductase-mediated gene amplification on the expression of transfected immunoglobulin genes. *J. Immunol. 139:4232.*
21. **Beidler, C. B., J. R. Ludwig, J. Cardenas, J. Phelps, C. G. Papworth, E. Melcher, M. Sierzega, L. J. Myers, B. W. Unger, M. Fisher, G. S. David, and M. J. Johnson.** 1988. Cloning and high level expression of a chimeric antibody with specificity for human carcinoembryonic antigen. *J. Immunol. 141:4053.*
22. **Li, Y.-W., D. K. Lawrie, P. Thammana, G. P. Moore, and C. W. Shearman.** 1990. Construction, expression and characterization of a murine/human chimeric antibody with specificity for hepatitis B surface antigen. *Mol. Immunol. 27:303.*
23. **Steplewski, Z., L. K. Sun, C. W. Shearman, P. Daddona, and H. Koprowski.** 1988. Biological activity of human-mouse IgG1, IgG2, IgG3, and IgG4 chimeric monoclonal antibodies with antitumor specificity. *Proc. Natl. Acad. Sci. USA 85:4852.*
24. **Sahagan, B. G., H. Dorai, J. Saltzgaber-Muller, F. Toneguzzo, C. A. Guindon, S. P. Lilly, K. W. McDonald, D. V. Morrissey, B. A. Stone, G. L. Davis, P. K. McIntosh, and G. P. Moore.** 1986. A genetically engineered murine/human chimeric antibody with specificity for human tumor-associated antigen. *J. Immunol. 137:1066.*
25. **LoBuglio, A. F., R. H. Wheeler, J. Trang, A. Haynes, K. Rogers, E. B. Harvey, L. Sun, and M. B. Khazeli.** 1989. Mouse/human chimeric monoclonal antibody in man: kinetics and immune response. *Proc. Natl. Acad. Sci. USA 86:4220.*
26. **Kurrle, R., W. Seyfert, A. Trautwein, and F. R. Seiler.** 1986. T cell activation by CD3 antibodies. In *Leukocyte Typing II.* E. L. E. Reinherz, ed. Springer-Verlag, Berlin, p. 137.
27. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA 74:5463.*
28. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analyt. Biochem. 162:156.*
29. **Aviv, H. and P. Leder.** 1972. Purification of biologically active globin mRNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA 69:1408.*
30. **Kaartinen, M., G. M. Griffith, P. H. Hamlyn, A. F. Markham, K. Karylainen, J. L. T. Pelkonen, O. Makela, and C. Milstein.** 1983. Anti-oxazolone hybridomas and the structure of the oxazolone idiotype. *J. Immunol. 130:937.*
31. **Chen, E. Y., and P. H. Seaberg.** 1985. Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. *DNA 4:165.*
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. In *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 280.
33. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analyt. Biochem. 132:6.*
34. **Schrezenmeier, H., R. Kurrle, H. Wagner, and B. Fleischer.** 1985. Activation of human T lymphocytes. III. Triggering of bystander cytotoxicity in cytotoxic T cell clones by antibodies against the T3 antigen or by a calcium ionophore. *Eur. J. Immunol. 15:1019.*
35. **Seiler, F. R.** 1972. Lymphocytotoxicity test: relation of serological in vitro test results to prolongation of skin allograft survival in *M. fascicularis. Behring Inst. Mitteilungen 51:46.*
36. **Kabat, E. A., T. T. Wu, M. Reid-Miller, N. M. Perry, and K. S. Gottesman.** 1987. In *Sequences of Proteins of Immunological Interest.* U.S. Dept. of Health and Human Services, U.S. Government Printing Office, Washington, D.C.
37. **Faure, F., S. Jitsukawa, F. Triebel, and T. Hercend.** 1988. Characterization of human peripheral lymphocytes expressing the CD3-gamma/delta complex with anti-receptor monoclonal antibodies. *J. Immunol. 141:3357.*
38. **Schlitt, H. J., R. Kurrle, and K. Wonigeit.** 1989. T cell activation by monoclonal antibodies directed to different epitopes on the human T cell receptor/CD3 complex: evidence for two different modes of activation. *Eur. J. Immunol. 19:1649.*
39. **Kurrle, R., H. U. Schorlemmer, C. W. Shearman, L. Lauffer, K. Frank, E. J. Kanzy, and F. R. Seiler.** 1991. Analysis of the immunoregulatory capacity of anti-α/β-TCR and anti-CD3 monoclonal antibodies. *Transplant. Proc. In press.*
40. **Smith, C. A., G. T. Williams, R. Kingston, E. J. Jenkinson, and J. J. T. Owen.** 1989. Antibodies to CD3/T cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature 337:181.*
41. **McConkey, D. J., P. Hartzell, J. F. Amador-Perez, S. Orrenius, and M. Jondal.** 1989. Calcium-dependent killing of immature thymocytes by stimulation via the CD3/T cell receptor complex. *J. Immunol. 143:1801.*

Cytotechnology 5: 47–55, 1991.

Production of recombinant proteins in serum-free media

D. Broad, R. Boraston and M. Rhodes
Celltech Ltd., 216 Bath Road, Slough SL1 4EN, UK

Key words: recombinant DNA, biopharmaceutical proteins, perfusion culture, fed-batch culture, serum-free media

Abstract

The advantages of serum-free culture for the manufacture of recombinant biopharmaceuticals from mammalian cells are reviewed. The process favoured is fed-batch serum-free cell culture. This process is applicable to the majority of cell lines, is practical on the large scale, gives the lowest manufacturing cost, and can be carried out without the use of any serum.

Introduction

The advent of recombinant DNA technology in 1976 heralded a new era in the pharmaceutical industry, by making it possible for the first time to manufacture many human proteins for therapeutic use. Initially it appeared that the bacterium *E. coli* would be the organism of choice for production of hormones such as somatomedin, human insulin and human growth hormone. However, it gradually became clear that bacteria are incapable of correct post-translational processing of polypeptides to generate more complex, authentic proteins (Arathoon and Birch, 1986; Rhodes and Birch, 1988). In particular, the inability of bacteria to correctly form disulphide bonds was a problem. This, however, could be achieved in some cases by solubilisation and refolding *in vitro*. More recently, secretion of correctly folded proteins from yeast or *E. coli* has been possible. The identification of complex glycoproteins of potential therapeutic value, however, resulted in the need to use mammalian cells capable of glycosylation.

There are now three recombinant proteins, manufactured using mammalian cells, which are marketed for treatment of humans. The first of these to be approved was tissue plasminogen activator (tPA), which has been licensed for treatment of myocardial infarction (Pennica *et al.*, 1983). The second recombinant mammalian cell product to be licensed was human growth hormone although it is not a glycoprotein. It is being used for replacement therapy in hypopituitary dwarfism (Carter *et al.*, 1988, Friedman *et al.*, 1989). Most recently, recombinant human erythropoetin has been approved for the treatment of anaemia in kidney transplantation (Davis *et al.*, 1987). Details of the actual manufacturing processes used have not been published and it is not known whether serum-free media are used commercially. This article, will therefore review some of the available published information on the principles of mammalian cell culture for production of these and other proteins in serum-free media.

Reasons for use of serum-free media

From the point of view of the manufacture of a therapeutic protein, the priorities are: safety, efficacy, consistency and cost of manufacture. The use of serum-free medium can assist the attainment of the required standards in a number of different ways. From the safety perspective we can see that elimination of serum from the culture medium can reduce the chance of contamination of the product by infectious microorganisms which may be present in the serum, or by serum proteins (Nguyen *et al.*, 1989). This in practice means that when serum-free media are used there is a reduction in cost of manufacture. The need for virus testing of serum is eliminated, and in-process testing of cell culture fluid is reduced, fewer purification steps may be needed, improving product recovery. If serum is used in media for the production of antibodies then it is necessary to use a serum with low contaminating IgG content to ease purification. This is usually foetal calf serum, which is expensive. Its omission will therefore reduce medium costs. (Nguyen *et al.*, 1989).

The efficacy of a product will be primarily determined by its structural integrity. The most common problem with protein products is proteolytic cleavage, and serum proteases such as plasmin may degrade sensitive proteins such as tPA (Lubiniecki *et al.*, 1989) and Pro-urokinase giving 'two-chain' forms (Avgerinos *et al.*, 1990). Again, because the quality of pharmaceutical products is strictly controlled, this means that improved cell culture yields and reduced purification losses may be encountered when serum-free media are used for production.

Consistency of production is greatly aided by the use of serum-free media, because the variability of composition of serum is avoided. This helps to assure the consistent purity and potency of biological products.

The overall effect of these benefits is to reduce the unit manufacturing cost of a product of given quality when serum-free medium is used. It is important when developing a biopharmaceutical product to select a serum-free medium as early as possible in the programme. Switching from one medium to another may affect the properties of the product, necessitating biochemical analysis, pharmacology and clinical testing to demonstrate equivalence.

Expression systems

A number of continuous mammalian cell lines have been employed for expression of recombinant proteins. The most commonly used have been the mouse mammary tumour cell line C127, the Chinese Hamster Ovary line (CHO), rodent myeloma lines, and the baby hamster kidney line (BHK) (Table 1). Less commonly used have been the Vero cell line and human lines such as HeLa or the Namalwa lymphoblastoid line.

A detailed discussion of the development of expression vectors, and their applications is beyond the scope of this article. However, the general principles will be outlined here. For further details see Bebbington and Hentschel (1985). An early system for introducing multiple copies of a gene into mammalian cells was the use of vectors based on the bovine papilloma virus (BPV). These may exist in multiple copies per nucleus in an episomic form. Integration into the chromosomes is also possible, again the occurrence of very high copy numbers is observed through random duplication.

Another method of gene amplification relies on the use of a dihydrofolate reductase (DHFR) deficient cell line, into which is transfected a vector which carries the DHFR gene. Selection of clones resistant to the folate antagonist, methotrexate, gives rise to populations containing multiple copies of the vector, which consequently produce greater amounts of the gene product (Schimke, 1984). A variation on this method employs the glutamine synthetase gene, and the toxic analogue methionine sulphoxamine.

A variety of promoters have been examined for the ability to direct high level transcription of mammalian genes eg. metallothionein promoter or strong viral promoters such as the RSV LTR, the HCTH LTR, etc. In favourable cases, good

Table 1. Examples of recombinant proteins expressed in mammalian cells

Product	Cell line	Culture system	Serum-free media?	Reference
	CHO	Suspension	Yes	Lubiniecki *et al.*, 1989;
tPA	Myeloma	Suspension	Yes	Birch and Rhodes 1988.
	C127	Microcarriers	Yes	
Scu-PA	CHO	Microcarriers	No	Avgerinos *et al.*, 1990.
EPO	CHO	Attached to plastic	Yes	Lin 1984.
TIMP	CHO	Suspension	Yes	Field *et al.*, 1989.
Chimeric	CHO	Suspension	Yes	Rhodes, 1989;
Mab	Myeloma	Suspension	Yes	Field *et al.*, 1990.
FVIII	BHK21	Attached to plastic	Yes	Pavarani *et al.*, 1987;
	CHO	–	Yes	Faure *et al.*, 1989.
FIX	CHO	Attached to plastic	No	Kaufman *et al.*, 1986.
Gamma inteferon	CHO	Suspension	Yes	Hayter *et al.*, 1989.
	C127	Microcarriers	Yes	Sano *et al.*, 1989.
Gamma interferon	C127	Attached to plastic	–	Carter *et al.*, 1988.
hGH	CHO	Attached to plastic	Yes	Friedman *et al.*, 1989.
IL-2	BHK	Microcarriers	Yes	Wagner *et al.*, 1989.
beta interferon	CHO	Attached to plastic	No	McCormick *et al.*, 1984.
hepatitis B surface antigen	CHO	Attached to plastic	No	Michel *et al.*, 1985.
human anti-thrombin 111	CHO	Attached to plastic	No	Zettlmeissl *et al.*, 1987
EDF	BHK	Microcarriers	Yes	Wirth *et al.*, 1989.
Von Willebrand factor	CHO	Suspension	Yes	Murata *et al.*, 1988.
	CHO	Microcarriers	Yes	Mignot *et al.*, 1989.

expression can be obtained from a single copy of a gene. Presumably this relies on the fortuitous insertion of the vector into an actively transcribed region of the chromosome.

Both the choice of cell line, and the choice of expression vector, will affect the type of cell culture process chosen and the success of process development efforts. In particular, cell growth in an attached mode places constraints on the type of culture system used and on the maximum scale of a unit process.

Serum-free media

No serum-free medium for the growth of the anchorage-dependent C127 cell line has been reported. Most workers have, therefore, used a serum-containing medium for growth of cells, and then a serum-free 'maintenance' medium for the (separate) production phase, during which the desired product is secreted, but only limited cell proliferation is seen (Reddy *et al.*, 1985; Carter *et al.*, 1988).

CHO cell lines can be grown in suspension culture, or attached to plastic or other surfaces in serum-free media such as those developed by Ham and others (Ham, 1965). A mixture of DMEM and F12 has commonly been employed for recombinant lines (Avgerinos *et al.*, 1990). For anchorage-dependent culture, serum is usually included for the initial growth phase, and then removed for the production phase.

Rodent myeloma cells can be grown in suspension using media specifically developed for the purpose using long-established principles laid down by Eagle, Iscove, etc. (Higuchi, 1973; Barnes and Sato, 1980). The growth of these lines in serum-containing media, followed by change to serum-free medium would require a novel filtration, centrifugation or other cell separation step, in contrast to the case of anchorage-dependent

cell lines, where serum removal is generally simpler.

The other papers in this issue of *Cytotechnology* and other sources may be consulted for further details of serum-free media, their composition and development.

Cell culture processes

Two approaches are taken to developing serum-free production processes. For cells growing attached to a solid surface, the main approach is to exploit the finding that cells grown to high cell density in serum-containing medium appear to tolerate the transition to serum-free medium, usually maintaining product synthesis with limited growth (Kluft *et al.*, 1983). This approach is of course not totally serum-free because growth occurs in the presence of serum. The purification advantages of serum-free media are achieved, but the regulatory issues still have to be addressed. The other approach, used mainly for suspension cell culture, is to develop a serum-free medium capable of supporting both growth and production.

Attached culture

Attachment-dependent cells are normally grown in serum-containing medium, which is then exchanged for a serum-free production medium. The simplest culture vessels such as tissue culture flasks or roller bottles can be used, the medium being exchanged manually. Although this is mainly a laboratory process, commercial quantities of therapeutic products can be produced in this way. The advantage of using this type of process is that a new product can be obtained quickly since no further process development is needed. Production can be increased by simply using more flasks. However, these processes are expensive to operate because they are very labour intensive. Costs can be reduced by automation, using robotics, as we have done at Celltech. However, more cost-effective unit operation processes can be developed, in which the attachment dependent cells are grown on microcarriers in fermenters (Fig. 1). Labour costs are reduced by growing the cells in a single fermenter unit rather than in thousands of bottles (1 100 L fermenter is equivalent to several thousand roller bottles). The use of microcarrier culture is economically attractive for attachment-dependent cells but severe engineering and technical problems have to be overcome, particularly in the area of sterility maintenance.

One problem to be solved in scaling up microcarrier culture is to find a method for removing serum-containing medium without damaging the cells. The simplest procedure is to let the microcarriers settle under gravity, and pump out the supernatant serum-containing medium. In practice, it is necessary to wash the microcarriers several times to completely eliminate the serum. The number of washes needed would depend on

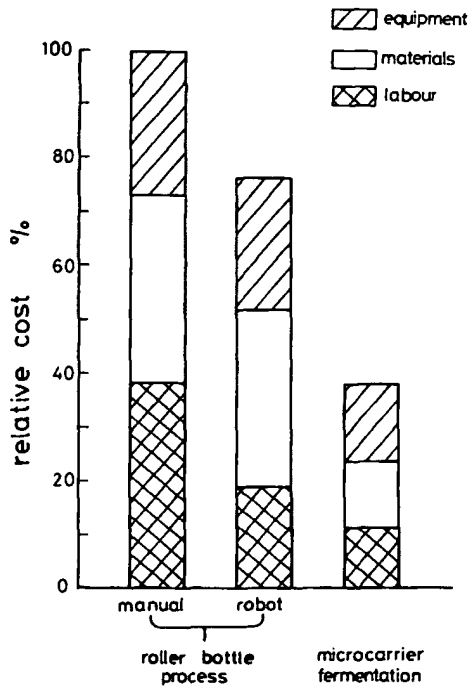


Fig. 1. The estimated direct Manufacturing costs for a mammalian cell biopharmaceutical product from three types of process for anchorage-dependent cells are compared.
Costs are per unit of active product.
Services, quality and building costs are equivalent.

Table 2. Perfusion culture methods

Biomass retention system	Reference
Spin filter	Himmelfarb *et al.* (1969)
Fluidized bed	Karkare S.B. *et al.* (1985)
Settling zone	Takazawa *et al.* (1988)
Hollow fibre	Knazek *et al.* (1972)

the initial concentration of serum and the volume of medium remaining within the cell/microcarrier bed. As scale increases, this procedure becomes less attractive, because settling times increase, leading to prolonged periods of depletion of nutrients, particularly oxygen. When the feasibility of this process was investigated at 120 L scale, it was found that up to 50% of the cells died during the medium exchange phase.

For this reason continuous perfusion culture systems have been developed in which the viability of the cells is maintained by continuously feeding in fresh serum-free medium whilst retaining the cells. This allows the serum to be diluted out and a continual stream of nutrient rich medium to be applied. A variety of techniques have been developed to do this (Table 2).

At Celltech we have developed perfusion culture systems for attachment-dependent cell lines utilizing spin-filters to retain the cells. An example of this process is shown in Fig. 2.

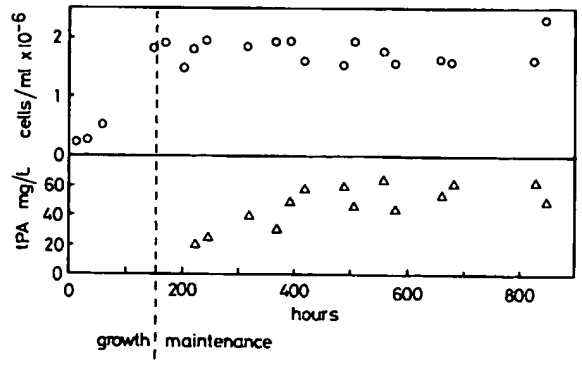


Fig. 2. Perfused microcarrier culture of C127 cells expressing tissue plasminogen activator (tPA). The cell line was grown in a serum-containing medium and maintained by perfusing with a proprietary protein-free tPA production medium. Perfusion rate was 0.5 fermenter volumes/day.

A process for producing tPA from C127 mouse fibroblast cells was scaled up to 40 L fermenter volume using a 30 day production phase of continuously perfused protein-free medium. This led to a tPA concentration of 55 mg/L (Fig. 2). We have now developed this technology further at 120 L fermenter volume scale, achieving greater than 4×10^7 cells/mL and a 32 day continually perfused protein-free production phase, with a recombinant CHO cell line.

Perfusion culture has also been applied to cells that can grow in suspension culture. However, it is not essential to do this because these cells can be economically grown in serum-free medium, allowing the technically simpler and more robust batch culture methods to be utilized.

Suspension culture

Mammalian cell recombinant DNA products are usually expressed in cell lines capable of growth in suspension – usually myelomas or CHO's. Celltech's approach has been to develop fermentation processes similar to those used successfully for the production of monoclonal antibodies from hybridomas. Cells are grown in serum-free suspension culture in airlift fermenters, with key nutrients and expression enhancers being added at pre-determined times during the fermentation to enhance both growth and specific productivity. We refer to such processes as fed batch culture. Using this technology, fermenter output can be increased several-fold, leading to reduced production costs.

Myeloma cells normally grow in suspension culture and it has been relatively straight forward to extend suspension fed-batch technology to

Table 3. Comparison of cellular productivity of CHO cells grown in attached or suspension culture in fermenters. For attached culture cells were inoculated onto Cytodex 2 microcarriers (5 g/L). In suspension culture the microcarriers were omitted. Specific cellular productivity is an average taken across the productive period

Cell line	Specific cellular productivity (product/cell/unit time)	
	Attached	Suspension
CHO r.protein 1	40	39
CHO r.protein 2	28	31

these cell types. Figure 3 illustrates a serum-free, fed-batch process developed for the production of tPA from a rat myeloma cell line (Kenten and Boss, 1985), yielding 56 mg/L of essentially single-chain tPA.

CHO cells can grow in suspension or attached culture mode. Usually the genetic manipulation of these cells is undertaken in attached culture; therefore they have to be 'adapted' to suspension growth. This is normally achieved by serial sub-culture in agitated culture systems such as Erlenmeyer flasks or spinner flasks (Faure *et al.*, 1989). Of key importance is that the cells do not lose productivity during the process – of all the CHO cell lines we have studied only one clone of a CHO-producing TIMP has been seen to lose productivity on adaption to suspension growth. This was not seen with other clones of the same cell line and thus remains a single, uncommon occurrence (Cockett *et al.*, 1990). Indeed when we have undertaken comparison of the CHO cells grown in fermenters, either in attached mode on microcarriers or in suspension culture, no differences in specific per cell productivity were seen (Table 3).

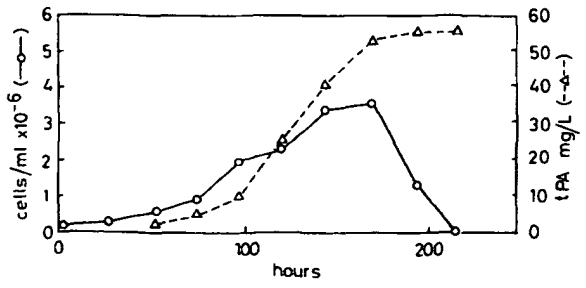


Fig. 3. Fed-batch airlift culture of myeloma cell line expressing tissue plasminogen activator (tPA). The myeloma cell line was grown in proprietary serum-free medium.

Once in suspension culture it is necessary to 'adapt' the cells to growth in serum-free medium. This is achieved by serial sub-culture and normally takes 1–4 weeks. Once again no reduction in cell productivity has been seen (Table 4). Media can be tailored to combine rapid adaption to both suspension and serum-free growth, reducing the adaption period to 10 days (Fig. 4).

Once cells are growing in serum-free suspension culture, cell banks are laid down. The productivity of the fermentation process is increased by feeding, at pre-determined times, key nutrients and enhancers to improve cell number and specif-

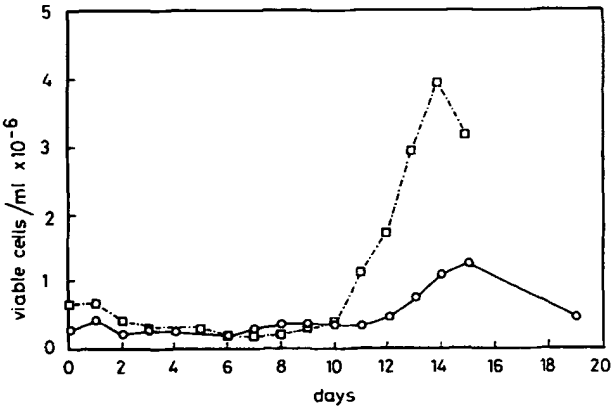


Fig. 4. 'Adaptation' of CHO cells to growth in serum-free, suspension culture. Cells were grown attached in T175 flasks, trypsinized and resuspended in 50 mL aliquots of medium in 250 mL Erlenmeyer flasks. These were agitated at 100 rpm and cell growth monitored by trypan blue exclusion. ○ Medium A – standard Celltech serum free medium. □ Medium B – medium developed at Celltech specifically for rapid adaption to serum-free, suspension growth.

Table 4. Specific cellular productivity of CHO cells grown in serum-containing or serum-free medium. Specific cellular productivity is an average taken across the productive period

System	Specific cellular productivity (product/cell/unit time)	
	+ Serum	– Serum
DHFR amplified		
CHO rec. protein 1	5.4	6.1
CHO rec. protein 2	11.6	9.9
GS amplified		
CHO rec. protein 3	10.8	8.2
CHO rec. protein 4	3.8	3.9
CHO rec. protein 5	4.9	6.2

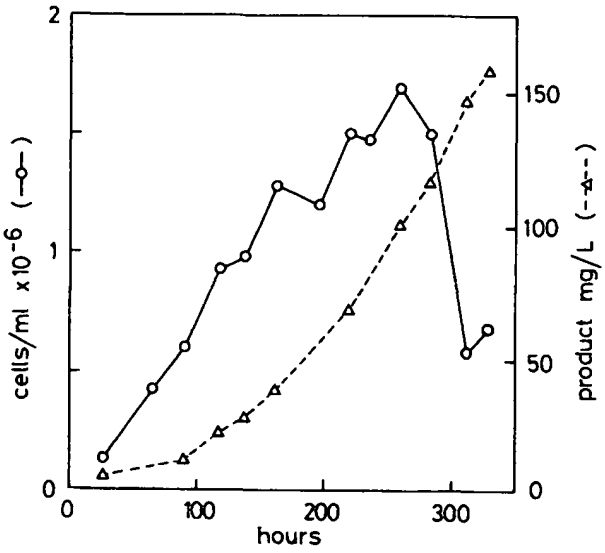


Fig. 5. Fed-batch fermentation of CHO cells producing a humanized Mab. O Viable cells; Δ Products.

ic per cell productivity. An example of what can be achieved is shown in Fig. 5, where the titre of a humanised recombinant antibody was increased four-fold to 160 mg/L by feeding nutrients to a CHO cell fermentation.

Conclusions

The applications of mammalian cell culture in the manufacture of biopharmaceutical products have progressed rapidly. While the use of serum-free culture is not essential, there are definite advantages in reducing manufacturing costs and ensuring a consistently high product quality.

Early applications of mammalian cell culture tended to use attached culture, involving the initial use of serum in the growth stage. This was then followed by a serum-free production stage. The most cost effective way of doing this is perfusion culture on microcarrier beads. More recently, the scale-up advantages of suspension culture have been generally recognised by the pharmacuetical industry, and several protein production processes have been developed in which the cells are grown in suspension in totally serum-free medium. Cell densities and protein productivity may be enhanced by carefully optimised nutrient feeding.

Thus serum-free medium will be a key feature of all modern cell culture processes for the pharmaceutical industry.

References

Arathoon WR and Birch JR (1985) Large scale cell culture in biotechnology. Science 232: 1390–1395.

Avgerinos GC, Drapeau D, Socolow JS, Mao J-I, Hsiao K and Broeze RJ (1990) Spin filter perfusion system for high density cell culture: production of recombinant urinary type plasminogen activator in CHO cells. Bio/technology 8: 54–58.

Barnes D and Sato G (1980) Serum-free cell culture: a unifying approach. Cell: 22: 649–655.

Bebbington C and Hentschel C (1985) The expression of recombinant DNA products in mammalian cells. Trends Biotechnol. 3: 314–317.

Carter MJ, Facklam TJ, Long PC, and Scotland PA (1988) Are continuous cell lines safe as substrates for human drugs and biologics? A case study with human growth hormone. Develop. Biol. Standard 70: 101–107.

Cockett MI, Bebbington, CR and Yarranton CT (1990) High level expression of Tissue inhibitor of metalloproteinase in Chinese Hamster Ovary cells using glutamine synthetase amplification. Biotechnology (in press).

Davis JM, Arakawa T, Strickland, TW and Yphantis DA (1987) Characterisation of recombinant human erythropoietin produced in Chinese Hamster Ovary cells. Biochemistry 26: 2633–2638.

Faure T, Meulien P, Kolbe HVJ, Mignot C and Pavirani A (1989) Engineering Foundation Conferences, New York, USA. The Development of expression systems for production of recombinant proteins in CHO cells: an example recombinant FV111. Cell Culture Engineering II p. 79.

Field R, Cockett M and Froud, SJ (1989) In: RE Spier, JB Griffiths, J Stephenne, PJ Crooy (eds.) Advances in Animal Cell Biology and Technology for Bioprocesses, pp. 195–197. Butterworths, Glutamine synthetase amplification of TIMP expression in CHO cells.

Field RP, Brand H, Renner GL, Robertson HA and Boraston R (1990) To be presented at European Society of Animal Cell Culture Technology meeting, May 1990. Production of chimeric antibody for tumour imaging and therapy from recombinant Chinese Hamster Ovary and myeloma cells (in press).

Friedman JS, Cofer GL, Anderson CL, Kushner JA, Gray PP, Chapman GE, Stuart MG, Lazarus L, Shine J and Kushner PJ (1989) High expression in mammalian cells without amplification. Bio/technology 7: 359–362.

Ham RG (1965) Clonal growth of mammalian cells in chemically defined synthetic media. Proceedings of National Academy of Sciences, USA 53: 288–293.

Hayter PM, Furlotte D, Wilcox M, Curling EMA, and Jenkins N (1989) In: RE Spier, JB Griffiths, J Stephanne, PJ Crooy (eds.) Advances in Animal Cell Biology and Technology for Bioprocess, pp. 280–282. Butterworths. Recombinant Gamma-Inteferon production by CHO cells in serum-free medium.

Higuchi K (1973) Cultivation of animal cells in chemically defined media, a review. Adv. Appl. Microbiol. 16: 111–136.

Himmelfarb P, Thayer PS and Martin HE (1969) Spin filter culture: The propagation of mammalian cells in suspension. Science 164: 555–557.

Karkare SB, Dean RC, Jr and Venkatasubramanian KJ (1985) Continuous fermentation with fluidized slurries of immobilized microorganisms. Bio/technology 3: 247–2.

Kaufman RJ, Wasley LC, Furie BC and Shoemaker CB (1986) Expression, purification, characterisation of recombinant γ carboxylated Factor IX synthesized in Chinese Hamster Ovary Cells. J. Biol. Chem. 261: 9622–9628.

Kenten J and Boss M (1985) Patent Application GB 86/00187, 4/1/85.

Kluft C, van Wezel AL, van der Velden CAM, Emeis JJ, Verheien JH and Wijngaards G (1983) In: Advances in Biotechnological Processes 2: 97–111. Large scale production of extrinsic (tissue type) plasminogen activator from human melanoma cells.

Knazek RA, Gullino PM, Kohler PO and Dedrick RC (1982) Cell culture on artificial capillaries. Science 178: 65.

Lin F-K (1984) European Patent Application 0148605. Production of Erythropoietin.

Lubiniecki A, Arathoon R, Plastri G, Thomas J, Wiebe M, Garnick R, Jones A, van Reis R and Builder S (1989) In: RE Spier, JB Griffiths, J Stephenne, PJ Crooy (eds.) Advances in Animal Cell Biology and Technology for Bioprocesses, pp. 442–449. Butterworths. Selected strategies for manufacture and control of recombinant tissue plasminogen activator prepared from cell cultures.

McCormick F, Trakey M, Innis M, Dieckmann B and Ringold G (1984) Inducible expression of amplified human beta interferon genes in CHO cells. Molecular Cellular Biology 4: 166–172.

Michel M-L, Sobczak E, Malpiece Y, Tiollais P and Shriek RE (1985) Expression of amplified hepatitis B virus surface antigen genes in Chinese Hamster Ovary Cells. Biotechnology 3: 561–566.

Mignot G, Ganne V, Faure T and van de Pol H (1989) Engineering Foundation Conferences, New York, USA. Production of recombinant von Willebrand factor in serum free culture by CHO cells grown on porous gelatin microcarriers. Cell Culture Engineering 11: p. 51.

Murata M, Eto Y and Shibai H (1988) Large-scale production of Erythroid Differentiation Factor (EDF) by gene-engineered Chinese Hamster Ovary (CHO) cells in suspension-culture. J. Fermentation Technology 66: 501–507.

Nguyen C, Rouchouse J and Pouradier-Duteil X (1989) In: RE Spier, JB Griffiths, J Stephenne, PJ Crooy (eds.) Advances in Animal Cell Biology and Technology for Bioprocesses, pp. 65–67. Butterworths. Contamination of Foetal Bovine Serum by Bovine Diarrhea Virus (BVD or MDV) and other related Pestiviruses.

Pavirani A, Meulien P and Hamer H, Schamber F, Dolf K, Villeval D, Cordier Y, Wiesel M-L, van de pol H, Piquet Y, Cazanove H-P and Le Coq J-P (1987) Choosing a host cell for active recombimant factor VIII production using vaccinia virus. Biotechnology 5: 389–392.

Pennica D, Kohr WJ, Harkins RN, Vehar GA, Ward CA, Bennett WF, Yelverton E, Seeburg PH, Heyneker HL and Goeddel DV (1983) Cloning and expression of human tissue – type plasminogen activator cDNA in *E. coli*. Nature 301: 214–221.

Reddy VB, Garramone AJ, Sasak H, Wei C, Watkins P, Galli J and Hsiung N (1985) Expression of human uterine tissue-type plasminogen activator in mouse cells using BPV vectors. DNA 6: 461–472.

Rhodes M and Birch J (1988) Large-scale production of proteins from mammalian cells. Bio/technology 6: 518–523.

Rhodes PM (1989) In: RE Spier, JB Griffiths, J Stephanne, PJ Crooy (eds.) Advances in Animal Cell Biology and Technology for Bioprocesses, pp. 472–474. Butterworths. Recombinant antibodies from CHO cells.

Sano E, Sawada R, Kasama K, Shimizu H and Kobayashi S (1989) Constitutive long term production of interferon-gamma by recombinant mouse C127 cells in serum-free medium – mouse C127 cell culture immobilized on microcarrier. J. Interferon Research 9: Suppl. 2, 5195.

Schimke RT (1984) Gene Amplification in cultured animal cells. Cell 37: 705–713.

Takazawa T, Takashiki M, Hamamoto K and Murakami H (1988) High cell density perfusion culture of hybridoma cells recycling high molecular weight components. Cytotechnology 1: 171–178.

Wagner R, Craft HJ and Lehmann J (1989) In: RE Spier, JB Griffiths, J Stephanne, PJ Crooy (eds.) Advances in Animal Cell Biology and Technology for Bioprocesses, pp. 374–376. Butterworths. The production of human interleukin 2 by recombinant mammalian cells.

Wirth M, Li S-Y, Lechmann J, Zettlmeissl G and Hauser H (1989) In: RE Spier, JB Griffiths, J Stephenne, PJ Crooy (eds.) Advances in Animal Cell Biology and Technology for Bioprocesses, pp. 44–50. Butterworths. Screening for and fermentation of high producer cell clones from recombinant BHK cells.

Wyatt DE and Dilling RR (1989) In: RE Spier, JB Griffiths, J Stephenne, PJ Crooy (eds.) Advances in Animal Cell Biology and Technology for Bioprocesses. Butterworths. Significance of raw material screening for custom-designed cell culture media.

Zebtlemeissel G, Ragg H and Karges HE (1987) Expression of biologically active human antithrombic III in Chinese Hamster Ovary Cells. Biotechnology 5: 720–725.

Address for offprints: D. Broad, Celltech Ltd., 216 Bath Road, Slough SL1 4EN, UK

ILL Document Delivery

VAUZGE

NLM -- W1 IN106D (GENCOLL)

US PATENT AND TRADEMARK OFFICE
SCIENTIFIC AND TECHNICAL INFO CTR
107 S. WEST STREET, PMB 803
ALEXANDRIA, VA 22314

ATTN:
PHONE: 571-272-2550
FAX: 571-272-0230
E-MAIL: STIC-DOCS@uspto.gov

SUBMITTED: 2007-01-30 14:56:43
PRINTED: 2007-01-31 10:26:34
REQUEST NO.: REG-14025529
SENT VIA: DOCLINE
DOCLINE NO: 21757663

REG **Copy** **Journal**

TITLE:	IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY : JOURNAL OF THE TISSUE CULTURE ASSOCIATION
PUBLISHER/PLACE:	Tissue Culture Association Gaithersburg Md
VOLUME/ISSUE/PAGES:	1986 Jul;22(7):363-8 363-8
DATE:	1986
AUTHOR OF ARTICLE:	Yabe N;Matsuya Y;Yamane I;Takada M
TITLE OF ARTICLE:	Enhanced formation of mouse hybridomas without hat
ISSN:	0883-8364
OTHER NUMBERS/LETTERS:	
	NLM Unique ID: 8506951
	PubMed ID: 3733634
SOURCE:	PubMed
MAX COST:	$4.00
COPYRIGHT COMP.:	Guidelines
CALL NUMBER:	W1 IN106D (GENCOLL)
REQUESTER INFO:	616635
DELIVERY:	E-mail Post to Web: STIC-DOCS@uspto.gov
REPLY:	Mail:

KEEP THIS RECEIPT TO RECONCILE WITH BILLING STATEMENT

For problems or questions, contact NLM at http://wwwcf.nlm.nih.gov/ill/ill_web_form.cfm or phone 301-496-5511.

Include LIBID and request number.

IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY
Volume 22, Number 7, July 1986

ENHANCED FORMATION OF MOUSE HYBRIDOMAS WITHOUT HAT TREATMENT IN A SERUM-FREE MEDIUM

NORITSUGU YABE, YUTAKA MATSUYA, ISAO YAMANE, AND MITSURU TAKADA

Department of Cell Biology, The Research Institute for Tuberculosis and Cancer, Tohoku University, 4-1 Seiryomachi, Sendai 980, Japan (N. Y., Y. M., I. Y.), and Department of Bacteriology, Yokohama City University School of Medicine, 2-33 Urafune, Minamiku, Yokohama 232, Japan (M. T.)

(Received 13 September 1985; accepted 17 January 1986)

SUMMARY

A newly developed, serum-free medium (NYSF-404) selects for antibody-producing hybridomas after fusion of antigen-sensitized mouse spleen cells with myeloma cell lines P3-X63-Ag8-U1 (P3-U1), P3-X63-Ag8-6.5.3 (Ag8.653), or P3-NSI/1-Ag4-1 (NS-1). Without the need for hypoxanthine-aminopterin-thymidine (HAT) selection of hybrid cells, frequency of hybridoma formation in medium NYSF-404 is higher (twice) than that in serum- and HAT-containing medium. Colonies developed upon limiting dilution in the presence of the mortal parent myeloma cells in medium NYSF-404 and pure culture of antibody-secreting cells could be subsequently established. The results suggest that fusions can be done in serum-free medium and that the clonal growth of hybridomas is dependent on factors produced by parent myeloma cells under serum-free culture conditions. Such factors seem deficient in serum- and HAT-containing medium or are masked by serum.

Key words: hybridoma; HAT-free selection; feeding effect; serum-free medium.

INTRODUCTION

The fusion of mortal antigen-sensitized spleen cells with immortal myeloma cells has been established for preparation of monoclonal antibodies that are specific to a variety of antigens (12,22). Despite the wide use of the technique, preparation of hybridomas is still not optimal. To select hybrid cells when a hypoxanthine phosphoribosyltransferase (HPRT)- or thymidine kinase-deficient myeloma cell line is used as a fusion partner, an addition of hypoxanthine-aminopterin-thymidine (HAT) to culture is required after a cell fusion (12,20). It seems likely that HAT selection eliminates the yield of hybridomas due to the instability of chromosomal retention (14,19) during an early period of the hybridoma formation or the inhibitory effect of thymidine on the proliferation of lymphocytes, in particular of human T-cell lines (7) for preparing T-T hybridomas (6).

On the other hand, an addition of serum to culture medium is necessary for in vitro proliferation of most cells. The degree of cell growth depends on the quality of serum; thus, a batch of serum should be carefully chosen because serum contains not only nutrient and growth-stimulating factors (9) but growth-inhibiting substances as well (16). Therefore, it is apparent that the preparation of sufficient quantities of hybridomas in serum-free culture is of importance.

Many advances have been introduced by others (1-4,10,15) in the serum-free culture of mouse hybridomas by supplementing basal media with low molecular weight compounds, hormones, transport proteins, lipids, or growth factors, or both. However, only a few investigators (1,10) have shown an application of serum-free but HAT-containing medium to the hybridoma formation.

We also developed the new serum-free medium, designated NYSF-404, which supports the growth of mouse hybridomas derived from a number of parent myeloma cell lines but not certain myeloma cell lines: P3-U1, Ag8.653, or NS-1 (in preparation). A development of such serum-free medium has led us to attempt a preparation of hybridomas without employing any additional supplements for the selection of hybrid cells and for cell growth.

MATERIALS AND METHODS

Cells and cell culture. Mouse HPRT-deficient myeloma cell lines P3-U1, Ag8.653, and NS-1 were routinely propagated in RPMI 1640 medium, containing 10% heat-inactivated fetal bovine serum (FBS) and 60 mg/l kanamycin sulfate at 37° C in a humidified atmosphere of 5% CO_2:95% air, without an adaptation to grow in serum-free medium before a cell fusion, and were occasionally cultured for a single passage in serum-containing RPMI 1640 supplemented with 8-azaguanine to eliminate potential revertants. The absence of

mycoplasma contaminants in these cell lines was confirmed by the fluorescent staining (17). Hybridomas developed in serum-free medium were maintained in both serum-containing RPMI 1640 and medium NYSF-404.

Preparation of serum-free culture medium NYSF-404. The sources of materials used in the preparation of medium NYSF-404 were as follows: Basal powdered media modified Eagle's minimum essential medium (MEM) and RPMI 1640 from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan; amino acids from Kyowa Hakko Kogyo Co., Ltd., Tokyo; hypoxanthine, thymidine, and bovine insulin from Sigma Chemical Co., St. Louis, MO; human iron-saturated transferrin from Alpha Therapeutic Corp., Los Angeles, CA; dihydroxyethylglycine (DHEG) from Dojindo Laboratories, Kumamoto, Japan; kanamycin sulfate from Meiji Seika Kaisha Ltd., Tokyo; and others from Wako Pure Chemicals Industries Co., Ltd., Tokyo. The composition of medium NYSF-404 is given in Table 1. Modified MEM is different from the original in omitting L-glutamine and sodium bicarbonate, and containing 75 mg/l succinic acid, 100 mg/l sodium succinate, 60 mg/l kanamycin sulfate (21), and 0.02 mg/l D-biotin.

After basal powdered media and buffer components had been dissolved in double distilled water, a mixed solution of other supplements prepared as 100-fold concentrate was added and then osmotic pressure of the medium was adjusted to 285 ± 5 mOsM/kg with DHEG. The medium was sterilized by membrane filtration through GV filter (0.22 μm, Millipore Corp. Bedford, MA), and stored in a tightly stoppered bottle at 4° C until use for up to 1 mo.

Other serum-free media based on MEM, RPMI 1640, and a 1:1 mixture of MEM and RPMI 1640 (MEM/RPMI) were supplemented with the same amounts of insulin, transferrin, sodium selenite, ethanolamine (15), and kanamycin sulfate in medium NYSF-404.

Cell fusion and hybridoma formation. A fusion ratio of spleen cells to myeloma cells varied from 2:1 to 10:1. Briefly, spleen cells (6×10^6 to 6×10^7) from BALB/c mice sensitized with C-reactive protein and myeloma cells (3×10^6 to 6×10^6) were fused with 50% polyethylene glycol (PEG) 4000 (E. Merck, Darmstad, West Germany) dissolved in serum-free RPMI 1640 for 2 min 3 d after the last injection of the antigen. The resulting cell pellet was divided into three equal parts. Each was reconstituted in a suspension with 20 ml of serum- and HAT-containing RPMI 1640, HAT-containing NYSF-404, or only NYSF-404, respectively. Approximately 200 μl of the cell suspension (2×10^4 to 4×10^5 cells) was added to each well of a 96-well microculture plate (Corning Glass Works, Corning, NY). Half of the culture supernatant in individual wells was renewed every 3 d. HAT-containing cultures were gradually replaced by HT-containing media from Day 14 of the fusion. On Day 21, the number of wells that contained growing colonies were counted and an antibody production assay was carried out.

Limiting dilution culture. A cloning of hybridomas developed in serum-free medium was done by limiting dilution with a minimum of one cell per well in 96-well microculture plates in medium NYSF-404. Before the limiting dilution culture started, the plates were seeded with peritoneal exudate cells, or thymocytes obtained from normal BALB/c mice, or parent myeloma cells, which had been successfully maintained in serum-containing RPMI 1640 and washed with serum-free RPMI 1640 to remove serum components, at a density of 5×10^4, 5×10^5, or 1×10^4 cells per well, respectively, as a feeder layer. The antibody production assay was carried out on Day 14 of the culture.

Antibody production assay. The supernatant in individual wells where colonies developed were screened by enzyme-linked immunosorbentassay as previously described (11).

Results

Efficient formation of hybridomas in medium NYSF-404. Based on the findings of our previous study, that medium NYSF-404 supports the growth of hybridomas but not of certain parent myeloma cell lines P3-U1, Ag8.653 and NS-1, we performed further investigation into whether hybridomas are able to form in serum-free

TABLE 1

COMPOSITION OF SERUM-FREE MEDIUM NYSF-404

Basal Media	
Modified Eagle's MEM	4560 mg/l
RPMI 1640 medium	5040
Amino Acids	
L-Arginine HCl	15
L-Asparagine H_2O	15
L-Glutamine	300
Glycine	5
L-Proline	5
L-Serine	15
L-Threonine	15
L-Valine	15
Vitamins, hormones, and trace element	
Vitamin B_{12}	0.00125
D-Biotin	0.0025
Human transferrin (holo)	10
Bovine insulin	10
Sodium selenite	0.0017
Other organic compounds	
Choline chloride	25
Glucose	500
Putrescine 2HCl	0.0125
Sodium pyruvate	110
Hypoxanthine	0.025
Thymidine	0.0125
Ethanolamine	20
Kanamycin sulfate	30
Buffers	
Dihydroxyethylglycine	1800
Sodium bicarbonate	1400

TABLE 2

ENHANCED FORMATION OF HYBRIDOMAS IN MEDIUM NYSF-404

Experiment No.	Fusion Ratio of Spleen Cells to Myeloma Cells[a]	Medium	HAT	No. of Wells With Growing Colonies	No. of Colonies per Well	No. of Wells With Antibody Production
1	2:1	RPMI + FBS[b]	+	38[c]	1-3	3
		NYSF-404	+	59	3-5	6
		NYSF-404	−	81	6-8	12
	10:1	RPMI + FBS	+	40	1-3	3
		NYSF-404	+	62	6-8	15
		NYSF-404	−	96	10<	18
2	2:1	RPMI + FBS	+	21	1-3	1
		NYSF-404	+	18	1-3	0
		NYSF-404	−	36	3-5	2
	10:1	RPMI + FBS	+	28	1-3	2
		NYSF-404	+	20	1-3	2
		NYSF-404	−	51	4-6	10

[a] 1×10^6 Myeloma cells (P3-U1 for experiment 1, Ag8.653 for experiment 2) and 2×10^6 or 1×10^7 spleen cells were treated with PEG per 96-well microculture plate.
[b] 10% Fetal bovine serum-containing RPMI 1640.
[c] Number of wells per 96-well microculture plate.

culture in medium NYSF-404 just after the cell fusion using these mortal parent myeloma cell lines. Table 2 shows that hybridomas derived from P3-U1 and Ag8.653 formed in medium NYSF-404. Rapidly growing colonies were observed within 5 d after the cell fusion in HAT-free NYSF-404, whereas colonies were generated several days later in HAT-containing media. In addition, the colony size was relatively larger in HAT-free culture (data not shown). The number of growing colonies and the yield of antibody-producing hybridomas markedly increased, at which a fusion ratio of spleen cells to myeloma cells rose from 2:1 to 10:1, even in the presence of HAT. The remarkable cell death by the overgrowth was not observed in both serum-containing RPMI 1640 and medium NYSF-404 even on Day 21 of the fusion. As shown in Table 3, hybridomas derived from NS-1 also formed effectively in medium NYSF-404.

Hybridomas were harvested most abundantly when the culture began at a density of 1×10^4 to 2×10^4 myeloma cells per well, whereas the yield decreased when more (5×10^4) or less (5×10^3) myeloma cells were employed (data not shown). Thus, a more rapid and efficient formation of hybridomas was obtained in medium NYSF-404 than in serum- and HAT-containing RPMI

TABLE 3

SUMMARIZED RESULTS OF HYBRIDOMA FORMATION OBTAINED IN SERUM-CONTAINING OR SERUM-FREE CULTURE

Myeloma Cells	Number of Experiments[a]	Medium	HAT	Percent of Wells With Growing Colonies[b]	Percent of Wells With Antibody Production[c]	Ratio of Antibody Production[d]
P3-U1	10	RPMI + FBS	+	33.0 ± 15.8[e]	7.3 ± 4.9	1.0
		NYSF-404	+	48.8 ± 17.5	8.8 ± 4.5	1.2
		NYSF-404	−	73.0 ± 14.4	12.1 ± 7.9	1.7
Ag8.653	4	RPMI + FBS	+	23.2 ± 11.6	5.0 ± 5.8	1.0
		NYSF-404	+	15.6 ± 6.6	2.6 ± 3.0	0.5
		NYSF-404	−	61.0 ± 10.7	14.0 ± 9.2	2.8
NS-1	3	RPMI + FBS	+	39.6 ± 15.6	8.4 ± 3.0	1.0
		NYSF-404	+	48.3 ± 17.5	7.9 ± 1.8	0.9
		NYSF-404	−	60.4 ± 9.0	18.3 ± 11.2	2.2

[a] 1×10^6 Myeloma cells and 5×10^6 spleen cells were treated with PEG per 96-well microculture plate in each fusion experiment.
[b] (No. of wells with growing colonies/total no. of seeded wells) × 100.
[c] (No. of wells with antibody production/no. of wells with growing colonies) × 100.
[d] In each medium to that in serum-containing RPMI 1640.
[e] Mean ± SD.

TABLE 4

DEVELOPMENT OF HYBRIDOMAS IN VARIOUS SERUM-FREE MEDIA WITH OR WITHOUT HAT

Experiment Number[a]	Medium	HAT	Number of Wells With Growing Colonies[b]	Number of Colonies Per Well	Number of Wells With Antibody Production
1	RPMI + FBS	+	23[b]	1-2	1
	MEM	−	10	1-2	1
	RPMI	−	14	1-2	1
	MEM/RPMI	+	20	1-2	1
	MEM/RPMI	−	27	2-4	5
	NYSF-404	+	21	1-3	1
	NYSF-404	−	58	4-6	9
2	RPMI + FBS	+	38	1-2	2
	MEM	−	24	1-3	1
	RPMI	−	18	1-2	0
	MEM/RPMI	+	40	2-4	5
	MEM/RPMI	−	44	2-4	7
	NYSF-404	+	61	6-8	4
	NYSF-404	−	48	4-6	8

[a] 1×10^6 Myeloma cells (P3-U1 for experiment 1, NS-1 for experiment 2) and 5×10^6 spleen cells were treated wtih PEG per 96-well microculture plate.
[b] Number of wells per 96-well microculture plate.

1640. Quite a few colonies developed from the mixed culture of spleen cells and myeloma cells which had been independently treated with PEG.

A formation of hybridomas derived from myeloma cell line Sp2/O-Ag14 (Sp2) or S194/5.XXO.BU.1 (S194) required HAT selection and a feeder layer, because these two myeloma cell lines could be propagated in medium NYSF-404 (unpublished results).

Table 3 summarizes the results obtained from the fusion experiments using three myeloma cell lines P3-U1, Ag8.653, and NS-1.

Feeding effect on myeloma cells on formation of hybridomas. Serum-free media based on MEM, RPMI 1640, and MEM/RPMI supported a development of colonies including antibody-producing hybridomas in the absence of HAT, although the frequency of colony formation was low (Table 4). However, it increased when each microculture was fed with additional parent myeloma cells (1×10^4), which are mortal in these serum-free media (data not shown).

The cultures with HAT maintained neither the clonal growth of cells nor cell survival, because additional parent myeloma cells themselves did not survive under such culture conditions due to the inhibitory effect of aminopterin. An addition of peritoneal cells (5×10^4) or thymocytes (5×10^5) to each serum-free microculture in medium NYSF-404, with or without HAT, promoted the hybridoma formation.

Clonal growth of hybridomas in medium NYSF-404. The cells, formed in medium NYSF-404, containing antibody-producing hybridomas were inoculated in microculture plates at a density of one cell per well. Table 5 shows that colonies including antibody-producing hybridomas were visible by 2 wk if the culture was fed with peritoneal exudate cells, thymocytes, or myeloma cells in medium NYSF-404. Furthermore, this serum-free culture system promoted an onset of the clonal growth more rapidly than serum-containing RPMI 1640.

Stability of hybridoma growth. Serum-free culture in medium NYSF-404 enabled cloned hybridomas to overcome poor culture environment for their growth when they were removed from microculture wells to larger vessels without an adaptation to the new environmental culture conditions. The cloned hybridomas have been cultivated successfully up to, at least, 7 mo. in medium NYSF-404, and almost all of them (three in four clones) continued to secret antibodies.

Parallel cultures of hybridomas in both serum-containing RPMI 1640 and medium NYSF-404 proved that there was no remarkable difference in the degree of antibody secretion during a period of the culture (data not shown).

DISCUSSION

Our serum-free medium NYSF-404 supports the excellent growth of a number of hybridomas derived from various mouse myeloma cell lines including Sp2 and S194, but not of certain parent myeloma cell lines P3-U1, Ag8.653, and NS-1 (in preparation), which suggested that

TABLE 5

EFFECT OF FEEDER CELLS ON CLONAL GROWTH OF HYBRIDOMAS IN SERUM-CONTAINING RPMI 1640 AND MEDIUM NYSF-404

Medium	Feeder Cells[a]	Number of Antibody-Positive Wells[b] A[c]	B	C	D
RPMI + FBS	None	0 (2)	0 (0)	0 (0)	
	Peritoneal cells	8 (44)	ND[d]	ND	
	Thymocytes	2 (29)	2 (19)	0 (0)	
NYSF-404	None	0 (0)	0 (1)	0 (1)	0 (0)
	Peritoneal cells	7 (39)	ND	ND	ND
	Thymocytes	9 (44)	7 (59)	5 (26)	8 (38)
	P3-U1				2 (27)
	NS-1				7 (33)

[a] The culture was fed with 5×10^4 peritoneal cells, 5×10^5 thymocytes, or 1×10^4 myeloma cells, respectively, per well.
[b] Number of wells per 96-well microculture plate containing specific antibody-producing cells, when the cells were seeded at a density of one cell per well. In parenthesis; number of wells with the clonal growth.
[c] Hybridomas derived from P3-U1 in column A, Ag8.653 in column B and NS-1 in columns C and D, respectively, were used in the limiting dilution culture.
[d] ND = not done.

we apply this medium to high yield of the hybridoma formation just after the cell fusion. In our preliminary experiments, serum- and HAT-containing culture without feeder cells, such as peritoneal macrophages, thymocytes, or spleen cells obtained from normal animals, made less hybridoma formation (unpublished results). Therefore, we also concluded that a feeder layer is essential for the optimal preparation of hybridomas, as others (5,8,18) have described. In the present study, however, we did not employ the feeder layer, because such feeder cells enhanced the frequency of the hybridoma formation in both medium NYSF-404 and serum- and HAT-containing RPMI 1640.

The yields of growing colonies and antibody-producing hybridomas increase when the fusion ratio of spleen cells to myeloma cells rises from 2:1 to 10:1 in the serum-free culture. It is probable that the augmentation is mainly due to the higher frequency at which myeloma cells encounter spleen cells, but not due to the feeding effect of the increasing number of PEG-treated spleen cells that are not fused with myeloma cells on the growth of hybridomas, because PEG-treated spleen cells do not survive any longer in culture.

One may point out that the low yield of hybridomas, in serum-containing culture for 21 d, is due to either unoptimal conditions of PEG-treatment (13) or the death of hybridomas caused by the overgrowth. However, we should emphasize that PEG exposure period of 2 min is optimal in our fusion system (unpublished results), and the cell death was negligible in not only serum-containing RPMI 1640 but medium NYSF-404, showing the development of hybridoma colonies is slower in serum-containing RPMI 1640 than in medium NYSF-404.

Kawamoto et al. (10) have reported that oleic acid added with fatty acid-free serum albumin is essential for the preparation of NS-1-derived hybridomas in their serum-free KSLM medium consisting of a 2:1:1 mixture of RPMI 1640, Dulbecco's modified Eagle's medium, and Ham's F12 supplemented with transferrin, insulin, selenium, ethanolamine, 2-mercaptoethanol, oleic acid, and low density lipoprotein. However, according to the authors, lipid supplement is not necessary for the efficient formation of hybridomas derived from NS-1-503, which is a clonal cell line isolated from NS-1 in lipid-deficient KSLM medium.

To the contrary, our present study proves that NS-1-derived hybridomas directly develop without requiring an adaptation period to lipid-free culture conditions in medium NYSF-404. This finding suggests that the efficient formation of hybridomas does not inure from nutrients provided from culture medium. As shown in the results involving serum-free medium based on MEM, it is also indicated that for an initial development of hybridomas, the feeder layers (including parent myeloma cells) are necessary rather than nutrients in medium.

The most critical phase of the hybridoma growth is when the cells are sparsely inoculated, e.g., during a clonal growth by the limiting dilution culture. It is obvious that the clonal growth of hybridomas is dependent not only on conventional feeder cells, but on parent myeloma cells that may provide undefined factors for an initial development of hybridomas in serum-free medium NYSF-404. Furthermore, the clonal growth is accelerated in NYSF-404 rather than in serum-containing RPMI 1640.

An alternative explanation for the efficient formation of hybridomas is that hybrid cells may be rescued, in HAT-free culture, from a death caused by a segregation of spleen X chromosome encoding active HPRT locus. Recently, Taggart and Samloff (19) have shown that such a low recovery of hybridomas due to instability of chromosomal retention is checked with the use of spleen cells of Robertsonian (8, 12) 5 Br mouse and HPRT- and adenosine phosphoribosyltransferase-deficient myeloma cell line FOX-NY. However, it must be stressed that, in our serum- and HAT-free culture, parent cell lines are not always deficient in marker enzymes that are related with pathways of DNA synthesis and responsible for a low recovery of resultant hybridomas.

The results strongly suggest that the formation and clonal growth of hybridomas depend on a feeding function of the mortal parent myeloma cells under serum-free culture conditions but not on serum factors. Thus, the application of serum-free medium to hybridoma technology would liberate us from a tedious and time-consuming procedure from the start of the fusion to a cloning and an expansion culture of hybridomas. Finally, these observations may have an important technical implication for the preparation of human-to-human hybridomas.

REFERENCES

1. Baker, P.; Knoblock, K.; Noll, L., et al. A serum independent medium effective in all aspects of hybridoma technology and immunological applications. In: Spier, R.; Griffiths, B.; Hennessen, W., eds. Developmental Biology Standard, vol. 60. Basel, Switzerland: Karger; 1985:63–72.
2. Chang, T. H.; Steplewski, Z.; Koprowski, H. Production of monoclonal antibodies in serum-free medium. J. Immunol. Methods 39:369–375; 1980.
3. Cleveland, W. L.; Wood, I.; Erlanger, B. F. Routine large-scale production of monoclonal antibodies in a protein-free culture medium. J. Immunol. Methods 56:211–234; 1983.
4. Darfler, F. J.; Insel, P. A. Serum-free culture of resting, PHA-stimulated and transformed lymphoid cells, including hybridomas. Exp. Cell Res. 138:287–295; 1983.
5. Fazekas de St. Groth, S.; Scheidegger, D. Production of monoclonal antibodies: Strategy and tactics. J. Immunol. Methods 35:1–21; 1980.
6. Foung, S. K. H.; Sasaki, D. T.; Grumet, F. C., et al. Production of functional human T-T hybridomas in selection medium lacking aminopterin and thymidine. Proc. Natl. Acad. Sci. USA 79:7484–7488; 1982.
7. Fox, R. M.; Tripp, E. H.; Tattersal, M. H. N. Mechanism of deoxycytidine rescue of thymidine toxicity in human T-leukemic lymphocytes. Cancer Res. 40:1718–1721; 1980.
8. Goding, J. W. Antibody production by hybridomas. J. Immunol. Methods 39:285–308; 1980.
9. Gospodarowicz, D.; Moran, J. S. Growth factors in mammalian cell culture. Annu. Rev. Biochem. 45:531–558; 1976.
10. Kawamoto, T.; Sato, J. D.; Le, A., et al. Development of a serum-free medium for growth of NS-1 mouse myeloma cells

and its application to the isolation of NS-1 hybridomas. Anal. Biochem. 130:445–453; 1983.
11. Köhler, G. Enzyme-immune assay and radio immunoassay. Hybridoma Techniques. Cold Spring Harbor, NY: Cold Spring Laboratory; 1980:21–30.
12. Kohler, G.; Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497; 1975.
13. Lane, R. D. A short-duration polyethylene glycol fusion technique for increasing production of monoclonal antibody-secreting hybridomas. J. Immunol. Methods 81:223–228; 1985.
14. Littlefield, J. W. Selection of hybrid from matings of fibroblast in vitro and their presumed recombinants. Science 145:709–710; 1964.
15. Murakami, H.; Masui, H.; Sato, G. H., et al. Growth of hybridoma cells in serum-free medium: Ethanolamine is an essential component. Proc. Natl. Acad. Sci. USA 79:1158–1162; 1982.
16. Price, P. J.; Gregory, E. A. Relationship between in vitro growth promotion and biophysical and biochemical properties of the serum supplement. In Vitro 18:576–584; 1982.
17. Russel, W. C.; Newman, C.; Williamson, D. H. A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasmas and viruses. Nature 253:461–462; 1975.
18. Russel, W. C.; Patel, G.; Precious, B., et al. Monoclonal antibodies against adenovirus type 5: Preparation and preliminary characterization. J. Gen. Virol. 56:393–408; 1981.
19. Taggart, R. T.; Samloff, I. M. Stable antibody-producing murine hybridomas. Science 219:1228–1230; 1983.
20. Trowbridge, I. S. Interspecies spleen-myeloma hybrid producing monoclonal antibodies against mouse lymphocyte surface glycoprotein, T200. J. Exp. Med. 148:313–323; 1978.
21. Yamane, I.; Matsuya, Y.; Jimbo, K. An autoclavable powdered culture medium for mammalian cells. Proc. Soc. Exp. Biol. Med. 127:335–336; 1968.
22. Yelton, D. E.; Margulies, D. H.; Diamond, B., et al. Plasmacytomas and hybridomas, development and applications. In: Kennet, R. H.; McKearn, T. J.; Bechtol, K. B., eds. Monoclonal antibodies: Hybridomas, a new dimension in biological analyses. New York: Plenum Press; 1980:8–17.

ADVANCES IN ANIMAL CELL BIOLOGY AND TECHNOLOGY FOR BIOPROCESSES

EDITORS
R.E.SPIER
J.B.GRIFFITHS
J.STEPHENNE
P.J.CROOY

BUTTERWORTHS

PART OF REED INTERNATIONAL P.L.C.

First published 1989

British Library Cataloguing in Publication Data

European Society for Animal Cell Technology,
(*Meeting: 9th: 1988: Knokke, Belgium*)
Advances in animal cell biology for bioprocesses
1. Animals. Cells. Culture. Techniques
I. Title II. Spier, R. E. (Raymond E.)
591'.0724

ISBN 0-407-01499-3

Library of Congress Cataloging-in-Publication Data

European Society of Animal Cell Technology.
General Meeting (9th: 1988: Knokke-Heist, Belgium)
Advances in animal cell biology and technology for bioprocesses/editors, R. E. Spier . . . [et al.].
p. cm.
"ESACT, European Society for Animal Cell Technology, the 9th Meeting."
Bibliography: p.
ISBN 0-407-01499-3 :
1. Animal cell biotechnology–Congresses. 2. Cytology–Congresses. I. Spier, R. (Raymond) II. Title.
~~TP248.27.A53E93 1988~~
660'.6–dc19

Printed and bound in Great Britain by
Courier International Ltd., Tiptree, Essex

Organiz

J. Stephenn
G. Charlier
P. Crooy
E. D'Hondt
P. d'Oultrer
L. Fabry
A. Miller
J. Werenne

GROWTH PROMOTING ABILITY OF HUMAN AND EGG TRANSFERRINS IN A SERUM FREE CULTURE SYSTEM

E. Bey, D. Botes,* S. Marrs,** R.L. Crookes ** and M. Shapiro**

Department of Microbiology, University of the Witwatersrand, Johannesburg; Department of Biochemistry, University of Cape Town * and S.A. Blood Transfusion Service, Johannesburg,** South Africa

ABSTRACT

The growth promoting properties of different preparations of human and egg transferrins were assessed using transferrin-dependent cells in serum-free, chemically defined medium with the aim of finding one most suited for large scale serum-free cell culture.

Various fractions of transferrin, prepared by column chromato- graphy, ultrafiltration and HPLC were tested in a microtest assy for growth promoting ability using a human B cell line (WI-L2), a human T cell line (Jurkat) and a mouse myeloma line (SP2/01-Ag 14) adapted to grow in serum-free medium (KSLMS).

Crude transferrin is toxic in the above cell culture systems; highly purified transferrin (99.99% pure) was as effective as commercial transferrin (97% pure). Egg transferrin had a delayed stimulatory effect on WI-L2 cells but did not support the growth of the other cell types.

INTRODUCTION

Transferrin is a glycoprotein containing two iron binding sites and has a MW of 76 kd. It is the major iron-binding protein of vertebrate serum (1).

The recognized functions of transferrin are as follows:

1. It facilitates the movement of biologically usable iron between the intacellular and the extracellular spaces.

2. Apotransferrin has a bacteriostatic and bacteriocidal function and is partly responsible for the self-sterilising ability of saliva, serum and other body fluids.

3. Transferrin plays a role in detoxification processes by removing toxic iron and other heavy metals.

4. It promotes cell growth.

This last aspect is discussed in the present investigation.

MATERIALS AND METHOD

Cell lines: Two human lymphoblastoid cell lines (WI-L2 and Jurkat) and one mouse myeloma line (Sp2/01-Ag 14) were used to measure the effects of human and egg transferrins of varying purity and iron content on cell division.

Medium: The experiments were carried out in KSLMS medium (2); the advantage of using serum-free, chemically defined medium is that it allows for identification of biologically active substances that might be relevant in regulation of tissue function (3)

Transferrin samples: A) Crude human transferrin obtained by column chromatography (92% pure), B) Highly purified fractions of transferrin obtained from the S.A. Blood Transfusion Service (99.99% pure), C) Highly purified ovotransferrin (99.99% pure), D) Commercial transferrin (97% pure)

10^3 cells/well were seeded into 24-well plates in KSLMS medium containing various fractions of human transferrin (1mg/l), ovotransferrin (1mg/l) and fetal calf serum (1%). Cell numbers were determined over 5 days using a Coulter Counter Model ZBI

RESULTS

1. None of the three cell lines grew in transferrin-free medium (Figs. 1 - 4)

2. Crude transferrin (92% pure) did not support cell growth and may be toxic in our cell culture system (Fig. 1)

3. Highly purified (99.9%) apo-, monoferric- and diferric-human transferrins support cell growth similarly to commercial transferrin (97% pure) (Figs. 2 - 4)

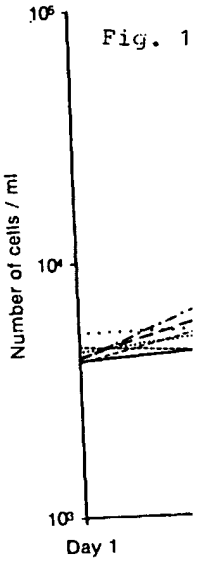
4. Highly purified ovotransferrin has a delayed stimulatory effect on WI-L2 cells but does not support the growth of other cell types (Figs. 2 - 4)

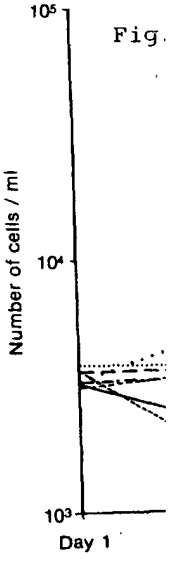
CONCLUSIONS

The results show that cell growth promoting ability of various human transferrin preparations is independent of iron content when they are above a certain degree of purity.

REFERENCES

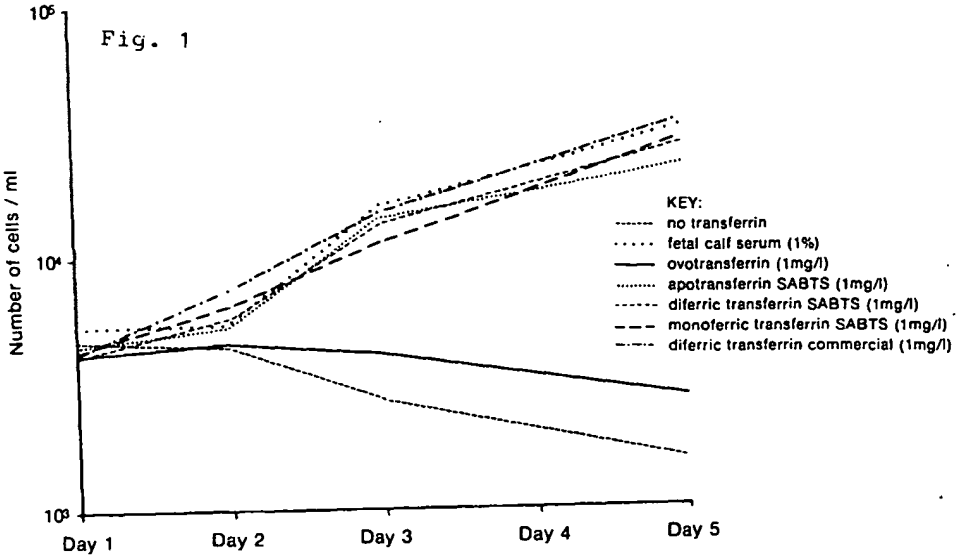
1. Morgan, E.H. (1981). Molecular Aspects of Med. Vol 4 pp 1-123.
2. Kawamoto,T., Sato,J.D., Le, A., McClure, D.B. and Sato, G.H. (1983). Anal Biochem. 130, pp 445-453.
3. Barnes, D. and Sato, G.H. (1980) Cell 22, pp 649-655.





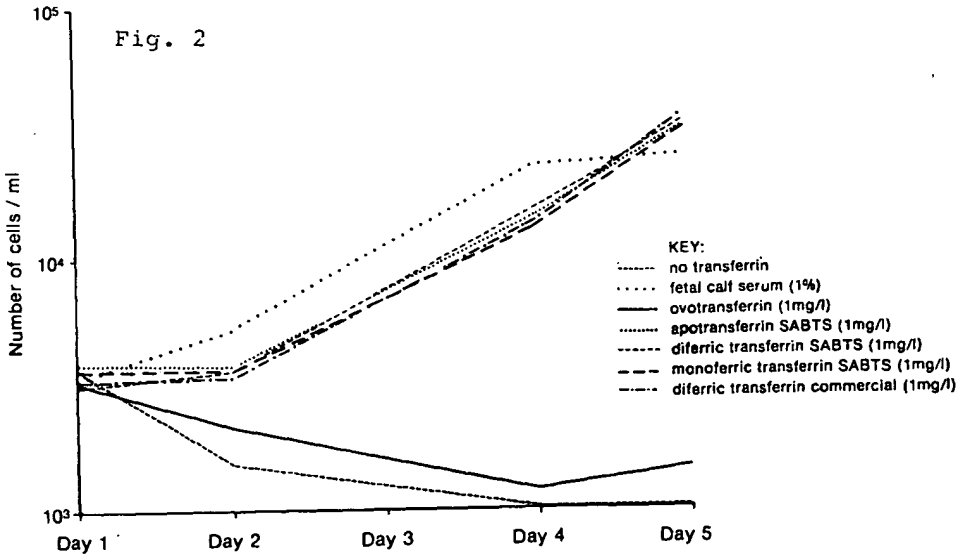
JURKAT

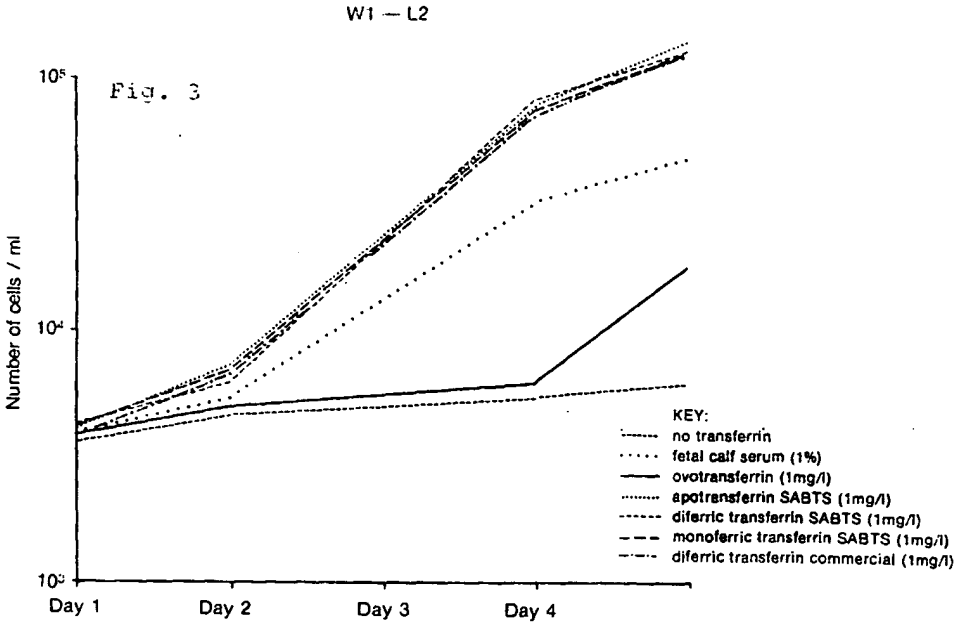
Fig. 1

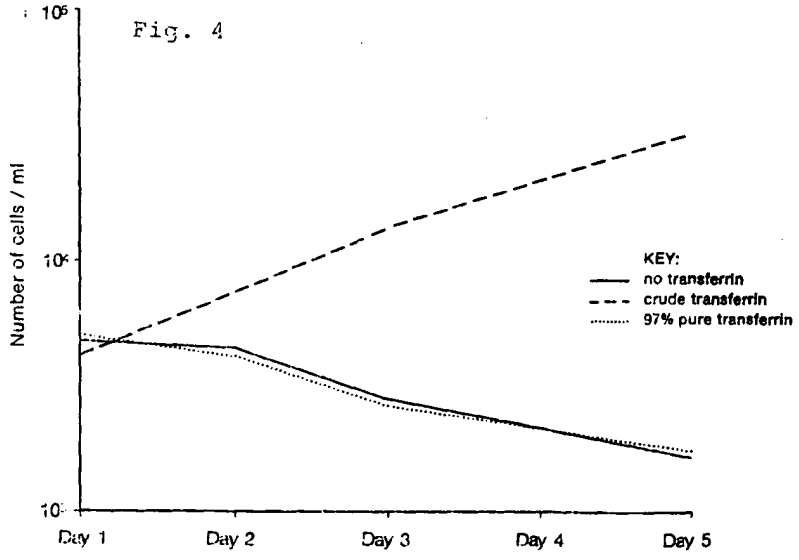


Sp2/01-Ag14

Fig. 2







The Adaptat
Monoclonal

Clark, S.A.
Technology
Applied Mic
U.K.

Abstract

The bulk in
defined, lo
objective o
protein ser
and a serum
three diffe
Growth and
static cult
and the res

Introductic

The product
quantities
been achiev
cavity of s
are generat
results in
normal muri
Although hy
usually the
final produ
When they a
impurities
acceptable,
of the anti
Looking for
however, it
the cells a
attractive.
our hybrido
free mediur

Methods

Media: The
(Imperial
inactivate
heat inact
Mass.). E
as a powde
supplement
filtration
The approx
medias was

of the C463A-derived rTNV148B production cell line designated C524A. rTNV148B is a totally human monoclonal antibody directed against TNFα, the genes for which were obtained using hybridoma techniques and transgenic mice.

Transfection and Screening

rTNV148B heavy chain expression vector, designated plasmid p1865, was linearized by digestion with XhoI and rTNV148B light chain expression vector, designated plasmid p1860, was linearized using SalI restriction enzyme. Approximately 1 x 10^7 C463A cells were transfected, with about 10μg of the premixed linearized plasmids, by electroporation (200 V and 1180 uF). *See* Knight et al., 30 MOLECULAR IMMUNOLOGY 1443 (1993). Following transfection, the cells were seeded at a viable cell density of 1 x 10^4 cells/well in 96-well tissue culture dishes with IMDM, 15% FBS, 2mM glutamine. After incubating the cells at 37°C, 5% CO_2 for about 40 hours, an equal volume of IMDM, 5% FBS, 2 mM glutamine and 2X MHX selection medium was added. The plates were incubated at 37°C, 5% CO_2 for about 2 weeks until colonies (primary transfectants) became visible.

Cell supernatants from wells in which there were visible colonies were assayed for human IgG by ELISA using a standard curve generated from protein-A column-purified rTNV148B human anti-TNF. Briefly, EIA plates (COSTAR®) were coated with 10 μg/ml of goat anti-human IgG Fc overnight at 4 C. After washing with 1X ELISA wash buffer (0.15 M NaCl, 0.02% Tween-20 (W/V)), the plates were incubated with about 50 µl of a 1:5 dilution of the 96-well supernatant for one hour at room temperature. After washing the plates with 1X ELISA wash buffer, alkaline phosphatase-conjugated goat anti-human IgG (heavy and light chains) (Jackson 109-055-088), and its substrate (Sigma® Aldrich 104-105), were used to detect the human IgG bound to the anti-Fc antibody coated on the plate.

Approximately one third of the colonies tested, i.e., the highest producers, were transferred to 24-well plates for further quantification and comparison of their expression levels. Cells were maintained in IMDM, 5% FBS, 2 mM glutamine and 1X MHX. Supernatants from spent 24-well cultures were assayed by ELISA as described above. The highest producing parental clones (primary transfectants) were identified based on the titers in 24-well spent cultures.

The seven top-producing clones were subcloned to identify a higher-producing, more homogeneous cell line. Ninety-six-well tissue culture dishes were seeded at 5 cells/ml and 20 cells/ml in IMDM, 5% FBS, 2mM glutamine and 1X MHX. The cells were incubated for about 14 days until colonies were visible. Cell supernatants from wells in which there

Ordered

ANSWER 60 OF 67 MEDLINE on STN DUPLICATE 37
ACCESSION NUMBER: 90258960 MEDLINE <<LOGINID::20070129>>
DOCUMENT NUMBER: PubMed ID: 2342491
TITLE: Construction, expression and characterization of a murine/human chimeric antibody with specificity for hepatitis B surface antigen.
AUTHOR: Li Y W; Lawrie D K; Thammana P; **Moore G P**; Shearman C W
CORPORATE SOURCE: Integrated Genetics, Inc., Framingham, MA 01701.
SOURCE: Molecular immunology, (1990 Mar) Vol. 27, No. 3, pp. 303-11.
Journal code: 7905289. ISSN: 0161-5890.
PUB. COUNTRY: ENGLAND: United Kingdom
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199006
ENTRY DATE: Entered STN: 20 Jul 1990
Last Updated on STN: 20 Jul 1990
Entered Medline: 28 Jun 1990

AB A murine/human chimeric antibody with specificity for Hepatitis B surface antigen has been produced by genetic engineering. The light and heavy chain variable region exons encoding the murine monoclonal antibody 2H1 were isolated and inserted into mammalian expression vectors containing the human kappa and gamma 1 constant region exons. The chimeric genes were transfected into murine **Sp2/0** hybridoma cells by electroporation and transfectomas secreting chimeric antibody were isolated. Secretion levels ranged from 1-7 pg/cell/24 hr. The chimeric antibody bound specifically to Hepatitis B surface antigen and competed effectively with the parental murine monoclonal antibody for binding to these sites. Chimeric 2H1 is the first clinically relevant, genetically engineered anti-viral antibody and may represent an improved agent for the prevention of hepatitis B virus transmission.

ANSWER 1 OF 43 MEDLINE on STN DUPLICATE 1
ACCESSION NUMBER: 2003450251 MEDLINE <<LOGINID::20070129>>
DOCUMENT NUMBER: PubMed ID: 14511573
TITLE: Adaptation of cell lines to serum-free culture medium.
AUTHOR: Ozturk S; Kaseko G; Mahaworasilpa T; Coster H G L
CORPORATE SOURCE: Research Institute for Genetic Engineering and Biotechnology, Tubitak Marmara Research Center, 41470 Gebze-Kocaeli, Turkey.. selma@rigeb.gov.tr
SOURCE: Hybridoma and hybridomics, (2003 Aug) Vol. 22, No. 4, pp. 267-72.
Journal code: 101131136. ISSN: 1536-8599.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200404
ENTRY DATE: Entered STN: 28 Sep 2003
Last Updated on STN: 16 Apr 2004
Entered Medline: 15 Apr 2004

AB The optimum conditions to allow proliferation of cells for the secretion of some growth factors and cytokines and the proliferation of cells in different media that do not contain proteins or serum from animals (serum-free media) were investigated. The culture of cell lines for the commercial production of products involves optimisation of cell proliferation and secretion in media from which the requisite proteins can be economically extracted. Some of these problems were addressed in this study. We used two different clones from a human **myeloma cell** line for adaptation to **serum free** medium in order to characterize long-term effects of the new medium. We gradually decreased serum content of medium and the results showed that cell mortality increased with serum reduction, antibody production lost by survived clones, and secretion of cytokines were always retained.

NSWER 16 OF 43 MEDLINE on STN DUPLICATE 5
ACCESSION NUMBER: 92136838 MEDLINE <<LOGINID::20070129>>
DOCUMENT NUMBER: PubMed ID: 1778091
TITLE: Cholesterol requirement for growth of IR983F and P3X63-Ag8-U1 **myeloma cells** in **serum-free** medium.
AUTHOR: Li J L; Li Y J; Chao Y J; Lin L X; Ouyang M H; Peng Y B; Chang W S
CORPORATE SOURCE: Department of Malaria Immunology, First Medical University of PLA, Guangzhou, The People's Republic of China.
SOURCE: Cytobios, (1991) Vol. 68, No. 272, pp. 15-22.
Journal code: 0207227. ISSN: 0011-4529.
PUB. COUNTRY: ENGLAND: United Kingdom
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199203
ENTRY DATE: Entered STN: 29 Mar 1992
Last Updated on STN: 3 Feb 1997
Entered Medline: 12 Mar 1992

AB Cholesterol, a major lipid component of the plasma membrane, is thought to have profound effects on the structure and function of cells. Most animal tissues are capable of synthesizing cholesterol de novo from acetate; however, there are relatively few mammalian cells in vitro expressing an absolute requirement for an exogenous source of cholesterol. In this paper, it was shown that both IR983F (983) rat myeloma cells and P3X63-Ag8-U1 (P3U1) mouse **myeloma cells** which had been cultivated in **serum-free** medium containing cholesterol for more than 6 months still required cholesterol in vitro for growth in serum-free medium. Optimal growth of 983 and P3U1 occurred in cholesterol concentrations of 15 and 5 micrograms/ml, respectively. Moreover, it was demonstrated that the cholesterol could be replaced by human low density lipoprotein in a concentration of 10 micrograms/ml but not by mevalonic acid lactone. In contrast to the parental myeloma cells, hybridoma cells derived from the mouse **myeloma cells** which had been cultivated in **serum-free** medium containing cholesterol for more than 6 months did not require cholesterol.

ANSWER 21 OF 43 BIOTECHDS COPYRIGHT 2007 THE THOMSON CORP. on STN
ACCESSION NUMBER: 1990-03432 BIOTECHDS <<LOGINID::20070129>>
TITLE: Growth promoting ability of human and egg transferrins in a serum-free culture system;
promotion of human B-lymphocyte WI-L2, T-lymphocyte Jurkat, mouse **myeloma cell** culture growth in **serum-free** culture medium with transferrin supplement (conference paper)
AUTHOR: Bey E; Botes D; Marrs S; Crookes R L; Shapiro M
LOCATION: Department of Microbiology, University of the Witwatersrand, Johannesburg, South Africa.
SOURCE: Adv.Anim.Cell Biol.Technol.Bioprocesses; (1989) 287-90
DOCUMENT TYPE: Journal
LANGUAGE: English
AN 1990-03432 BIOTECHDS <<LOGINID::20070129>>
AB Human lymphoblastoid cell lines WI-L2 and Jurkat and mouse myeloma SP2/01-Ag14 were used to measure the effects of human and egg transferrins of varying purity and iron content on cell divisions. The cells were seeded at a concentration of 1000 cells/well into 24-well plates in KSLMS serum-free culture medium containing 1 mg/l human transferrin, 1 mg/l ovatransferrin and 1% fetal cattle serum. None of the 3 cell lines grew in transferrin-free medium. Crude transferrin (92% pure) did not support cell growth and may be toxic in these cell culture systems. Human highly purified (99.9%) apo-transferrin, monoferric-transferrin and diferric-transferrin supported cell growth similarly to commercial transferrin (97% pure). Highly purified ovatransferrin had a delayed stimulatory effect on WI-L2 cells but did not support the growth of other cell types. The results showed that the cell growth promoting ability of various human transferrin preparations is independent of iron content when they are above a certain degree of purity. (3 ref)

ANSWER 20 OF 43 BIOTECHDS COPYRIGHT 2007 THE THOMSON CORP. on STN
ACCESSION NUMBER: 1990-03214 BIOTECHDS <<LOGINID::20070129>>
TITLE: Secretion of recombinant hybrid plasminogen-activators by mouse myeloma cells;
hybrid tissue plasminogen-activator, urokinase production by mouse **myeloma cell** culture in **serum-free** culture medium; gene amplification (conference paper)
AUTHOR: Pierard L; Garcia Quintana L; Reff M E; Bollen A
CORPORATE SOURCE: SK+F
LOCATION: Service de Genetique Appliquee, Universite Libre de Bruxelles, rue de l'Industrie 24, B-1400 Nivelles, Belgium.
SOURCE: Adv.Anim.Cell Biol.Technol.Bioprocesses; (1989) 475-80
DOCUMENT TYPE: Journal
LANGUAGE: English
AN 1990-03214 BIOTECHDS <<LOGINID::20070129>>
AB Genes encoding 4 different hybrids (tPPUK, K2UK, FgUK, FgK2UK), combining structural domains of tissue-type plasminogen-activator (t-PA) and urokinase (EC-3.4.21.31)-type plasminogen-activator (u-PA), were inserted into a plasmid pTND-based vector encoding dihydrofolate-reductase (DHFR, EC-1.5.1.3), neomycin-phosphotransferase and the protein of interest. The plasmids were introduced into myeloma P3X63Ag8.653 by electroporation. The best producing clones were exposed to DHFR co-amplification with methotrexate. Expression levels were usually raised to 4-5 pg/cell/day. The producer clones of each construction were grown in serum-free culture medium and the enzymes were concentrated by ultrafiltration. Natural u-PA, FgUK, K2UK, FgK2UK and tPPUK had mol.weight of 54,000, 58,000, 65,000, 67,000 and 69,000 (SDS-PAGE), respectively. The protease moiety of u-PA present in the hybrids was not altered. Hybrids FgUK, K2UK, FgK2UK had little or no fibrin-binding activity, while hybrid tPPUK had a lower fibrin binding capability than t-PA. (7 ref)

ANSWER 22 OF 43 BIOTECHDS COPYRIGHT 2007 THE THOMSON CORP. on STN
ACCESSION NUMBER: 1990-14686 BIOTECHDS <<LOGINID::20070129>>
TITLE: An affinity based isolation process for myeloma tPA; recombinant tissue plasminogen-activator production, purification method involving perfusion and metal chelate affinity chromatography; myeloma cell culture (conference paper)
AUTHOR: Callaway J
CORPORATE SOURCE: SK+F; SK-Beecham
LOCATION: Department of Biological Process Sciences, SmithKline French Research Laboratories, SmithKline Beecham, King of Prussia, PA 19406, USA.
SOURCE: Biotech USA; (1989) 231-36
DOCUMENT TYPE: Journal
LANGUAGE: English
AN 1990-14686 BIOTECHDS <<LOGINID::20070129>>
AB An isolation method was developed for a tissue plasminogen-activator (tPA) with the same primary sequence as the wild-type form. The molecule was expressed in a **myeloma cell** line and secreted into a **serum-free** culture medium using a perfusion system with a production cycle of almost 100 days. The recombinant tPA was recovered as 76-90% single chain form, but lacked detectable levels of sialic acid and possessed significantly reduced solubility near neutral pH as compared to Chinese hamster ovary (CHO)-derived tPA. The isolation process employs a metal chelate affinity capture step (chromatography using a Chelating Sepharose Fast Flow affinity resin 3-6 L column) followed by neutral pH precipitation. A tripeptide-based affinity step is used with a high ionic strength binding and wash conditions to remove trace contaminants. Size exclusion chromatography is used as a final purification step. The method is designed to meet regulations for clearance of DNA and adventitious agents such as viruses and produces 95% pure proteins. (5 ref)

ANSWER 33 OF 43 MEDLINE on STN DUPLICATE 7
ACCESSION NUMBER: 86277800 MEDLINE <<LOGINID::20070129>>
DOCUMENT NUMBER: PubMed ID: 3733634
TITLE: Enhanced formation of mouse hybridomas without hat treatment in a serum-free medium.
AUTHOR: Yabe N; Matsuya Y; Yamane I; Takada M
SOURCE: In vitro cellular & developmental biology : journal of the Tissue Culture Association, (1986 Jul) Vol. 22, No. 7, pp. 363-8.
Journal code: 8506951. ISSN: 0883-8364.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 198609
ENTRY DATE: Entered STN: 21 Mar 1990
Last Updated on STN: 3 Feb 1997
Entered Medline: 17 Sep 1986

AB A newly developed, serum-free medium (NYSF-404) selects for antibody-producing hybridomas after fusion of antigen-sensitized mouse spleen cells with myeloma cell lines P3-X63-Ag8-U1 (P3-U1), P3-X63-Ag8-6.5.3 (Ag8.653), or P3-NSI/1-Ag4-1 (NS-1). Without the need for hypoxanthine-aminopterin-thymidine (HAT) selection of hybrid cells, frequency of hybridoma formation in medium NYSF-404 is higher (twice) than that in serum- and HAT-containing medium. Colonies developed upon limiting dilution in the presence of the mortal parent myeloma cells in medium NYSF-404 and pure culture of antibody-secreting cells could be subsequently established. The results suggest that fusions can be done in serum-free medium and that the clonal growth of hybridomas is dependent on factors produced by parent **myeloma cells** under **serum-free** culture conditions. Such factors seem deficient in serum- and HAT-containing medium or are masked by serum.

ANSWER 2 OF 5 BIOTECHDS COPYRIGHT 2007 THE THOMSON CORP. on STN
ACCESSION NUMBER: 2003-20531 BIOTECHDS
TITLE: New myeloma cell line **C463A** and any cell line derived from it useful for production of proteins e.g. immunoglobulins, cytokines, growth factors, hormones, blood proteins, and antimicrobials;
recombinant protein production via cell culture for use in disease therapy
AUTHOR: LEE C; LY C; MOORE G; SAVINO E
PATENT ASSIGNEE: CENTOCOR INC
PATENT INFO: WO 2003051720 26 Jun 2003
APPLICATION INFO: WO 2002-US39605 11 Dec 2002
PRIORITY INFO: US 2001-339428 14 Dec 2001; US 2001-339428 14 Dec 2001
DOCUMENT TYPE: Patent
LANGUAGE: English
OTHER SOURCE: WPI: 2003-532986 [50]
AN 2003-20531 BIOTECHDS
AB DERWENT ABSTRACT:

ANSWER 28 OF 67 HCAPLUS COPYRIGHT 2007 ACS on STN
ACCESSION NUMBER: 2002:658225 HCAPLUS <<LOGINID::20070129>>
DOCUMENT NUMBER: 137:184584
TITLE: Chemically defined medium for cultured mammalian cells
INVENTOR(S): **Lee, Chichang**; Ly, Celia; **Moore, Gordon**; Perkinson, Robert
PATENT ASSIGNEE(S): Centocor, Inc., USA
SOURCE: PCT Int. Appl., 29 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2002066603	A2	20020829	WO 2002-US3274	20020205
WO 2002066603	A3	20021219		
W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG				
CA 2438148	A1	20020829	CA 2002-2438148	20020205
US 2003096402	A1	20030522	US 2002-67382	20020205
US 6900056	B2	20050531		
EP 1360314	A2	20031112	EP 2002-709335	20020205
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI, RO, MK, CY, AL, TR				
JP 2005505240	T	20050224	JP 2002-566310	20020205
PRIORITY APPLN. INFO.:			US 2001-268849P P	20010215
			WO 2002-US3274 W	20020205

AB The present invention relates to methods and compns. for chemical defined media for growth of mammalian cells for production of com. useful amts. of expressed proteins.

ANSWER 23 OF 67 HCAPLUS COPYRIGHT 2007 ACS on STN
ACCESSION NUMBER: 2003:696447 HCAPLUS <<LOGINID::20070129>>
DOCUMENT NUMBER: 139:213025
TITLE: Myeloma cell line useful for manufacturing recombinant proteins in chemically defined media
INVENTOR(S): **Lee, Chichang; Savino, Edward; Moore, Gordon**; Ly, Celia
PATENT ASSIGNEE(S): USA
SOURCE: U.S. Pat. Appl. Publ., 34 pp.
CODEN: USXXCO
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 2
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
US 2003166146	A1	20030904	US 2002-316308	20021211
WO 2003051720	A2	20030626	WO 2002-US39605	20021211
W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW				
RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SI, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG				
AU 2002351357	A1	20030630	AU 2002-351357	20021211
US 2004152170	A1	20040805	US 2003-727432	20031204

PRIORITY APPLN. INFO.:		
US 2001-339428P	P	20011214
US 2002-316308	A2	20021211
WO 2002-US39605	W	20021211

AB The present invention provides a novel myeloma cell line, designated **C463A**, and derivs. of **C463A**, which have the ability to grow continuously in chemical defined media. The present invention also relates to the production of proteins in cell line **C463A** and any cell line derived therefrom. The present invention further relates to methods for identifying cell lines capable of growing in chemical defined media. The present invention also relates to business methods where customers are provided with the cells, cell lines, and cell cultures of the present invention.

ANSWER 22 OF 67 HCAPLUS COPYRIGHT 2007 ACS on STN

ACCESSION NUMBER: 2003:377056 HCAPLUS <<LOGINID::20070129>>
DOCUMENT NUMBER: 138:384155
TITLE: Enhanced antibody expression and/or growth of cultured cells using co-transcription of human bcl2 protein
INVENTOR(S): **Lee, Chichang**; Ly, Celia; **Moore, Gordon**; **Shi, Xiaomei**
PATENT ASSIGNEE(S): Centocor, Inc., USA
SOURCE: PCT Int. Appl., 64 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2003040374	A1	20030515	WO 2001-US45553	20011102

W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM
RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG

PRIORITY APPLN. INFO.: WO 2001-US45553 20011102

AB The present invention in the field of biotechnol., provides methods and compns. for providing enhanced growth of, and/or protein production from, cultured mammalian host cells used for the production of com. useful amts. of expressed proteins, by the use of at least one Bcl2 encoding nucleic acid provided or transcribed in the host cell. Bcl2 clones were derived from two IgG producing myeloma cell lines and **Sp2/0** and all Bcl2 clones expressed the 29 KD human Bcl2 protein. All IgG producing Bcl2 clones maintained a high IgG expression phenotype. Bcl2 cultures survived twice as long as their parental cell cultures in optimal medium, but their cell d. was 20 to 50% lower than that of their parental cultures. L-glutamine/glucose feed did not improve the cell d. of the Bcl2 cultures. The IgG titer of the Bcl2 cultures was either close to or slightly higher than the titer of their parental cells even though their cell d. was generally much lower. Bcl2 cultures survived in **serum free**, animal protein stressed medium, while their parental cells could not. Bcl2 cultures were capable of resisting much higher ammonium concns. than their parental cells.

REFERENCE COUNT: 6 THERE ARE 6 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

ANSWER 21 OF 67 BIOTECHDS COPYRIGHT 2007 THE THOMSON CORP. on STN
ACCESSION NUMBER: 2003-20531 BIOTECHDS <<LOGINID::20070129>>
TITLE: New myeloma cell line **C463A** and any cell line derived from it useful for production of proteins e.g. immunoglobulins, cytokines, growth factors, hormones, blood proteins, and antimicrobials;
recombinant protein production via cell culture for use in disease therapy
AUTHOR: **LEE C**; LY C; **MOORE G**; **SAVINO E**
PATENT ASSIGNEE: CENTOCOR INC
PATENT INFO: WO 2003051720 26 Jun 2003
APPLICATION INFO: WO 2002-US39605 11 Dec 2002
PRIORITY INFO: US 2001-339428 14 Dec 2001; US 2001-339428 14 Dec 2001
DOCUMENT TYPE: Patent
LANGUAGE: English
OTHER SOURCE: WPI: 2003-532986 [50]
AN 2003-20531 BIOTECHDS <<LOGINID::20070129>>
AB DERWENT ABSTRACT:
NOVELTY - Myeloma cell line **C463A** and any cell line derived from it are new.

ANSWER 20 OF 67 BIOTECHDS COPYRIGHT 2007 THE THOMSON CORP. on STN
DUPLICATE 12
ACCESSION NUMBER: 2003-19861 BIOTECHDS <<LOGINID::20070129>>
TITLE: New clonal myeloma cell line or its derivative capable of growing continuously in a chemically defined medium, useful for producing recombinant therapeutic proteins at large scale ;

for use in recombinant protein preparation and cancer, bacterium, fungus and virus infection therapy

AUTHOR: **LEE C**; LY C; **MOORE G**; **SAVINO E**
PATENT ASSIGNEE: CENTOCOR INC
PATENT INFO: WO 2003052064 26 Jun 2003
APPLICATION INFO: WO 2002-US39496 11 Dec 2002
PRIORITY INFO: US 2001-339429 14 Dec 2001; US 2001-339429 14 Dec 2001
DOCUMENT TYPE: Patent
LANGUAGE: English
OTHER SOURCE: WPI: 2003-514045 [48]
AN 2003-19861 BIOTECHDS <<LOGINID::20070129>>
AB DERWENT ABSTRACT:

NSWER 14 OF 67 HCAPLUS COPYRIGHT 2007 ACS on STN
ACCESSION NUMBER: 2004:182243 HCAPLUS <<LOGINID::20070129>>
DOCUMENT NUMBER: 140:234390
TITLE: Methods and compositions for enhanced protein expression and/or growth of cultured cells using co-transcription of a bcl2 encoding nucleic acid
INVENTOR(S): **Lee, Chichang**; **Shi, Xiaomei**; Ly, Celia; **Moore, Gordon**
PATENT ASSIGNEE(S): USA
SOURCE: U.S. Pat. Appl. Publ., 42 pp.
CODEN: USXXCO
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
US 2004043028	A1	20040304	US 2001-3632	20011102
PRIORITY APPLN. INFO.:			US 2001-3632	20011102

AB The present invention in the field of biotechnol., provides methods and compns. for providing enhanced growth of, and/or protein production from, cultured mammalian host cells used for the production of com. useful amts. of expressed proteins, by the use of at least one Bcl2 encoding nucleic acid provided or transcribed in the host cell. Bcl2 clones were derived from two IgG producing myeloma cell lines and **Sp2/0** and all Bcl2 clones expressed the 29 kDa human Bcl2 protein. All IgG producing Bcl2 clones maintained a high IgG expression phenotype. Bcl2 cultures survived twice as long as their parental cell cultures in optimal medium, but their cell d. was 20 to 50% lower than that of their parental cultures. L-glutamine/glucose feed did not improve the cell d. of the Bcl2 cultures. The IgG titer of the Bcl2 cultures was either close to or slightly higher than the titer of their parental cells even though their cell d. was generally much lower. Bcl2 cultures survived in **serum free**, animal protein stressed medium, while their parental cells could not. Bcl2 cultures were capable of resisting much higher ammonium concns. than their parental cells.

ANSWER 41 OF 43 SCISEARCH COPYRIGHT (c) 2007 The Thomson Corporation on STN

ACCESSION NUMBER: 1984:219245 SCISEARCH <<LOGINID::20070129>>
THE GENUINE ARTICLE: SM671
TITLE: ADAPTATION OF A MURINE **MYELOMA CELL**
-LINE TO **SERUM-FREE** MEDIA AND ITS USE
AS A FUSION PARTNER IN MONOCLONAL-ANTIBODY PRODUCTION
AUTHOR: BROWN B L (Reprint); SHRIVER K E; HARSHMAN J S; RENER J C
CORPORATE SOURCE: HAZLETON BIOTECHNOL CORP, VIENNA, VA 22180
COUNTRY OF AUTHOR: USA
SOURCE: IN VITRO-JOURNAL OF THE TISSUE CULTURE ASSOCIATION, (1984) Vol. 20, No. 3, pp. 271-271.
ISSN: 0073-5655.
PUBLISHER: SOC IN VITRO BIOLOGY, 8815 CENTRE PARK DR,STE 210, COLUMBIA, MD 21045.
DOCUMENT TYPE: Conference; Journal
FILE SEGMENT: LIFE
LANGUAGE: English
REFERENCE COUNT: 0
ENTRY DATE: Entered STN: 1994
Last Updated on STN: 1994

ANSWER 40 OF 43 SCISEARCH COPYRIGHT (c) 2007 The Thomson Corporation on STN

ACCESSION NUMBER: 1984:258781 SCISEARCH <<LOGINID::20070129>>
THE GENUINE ARTICLE: SQ094
TITLE: HYBRIDOMA AND **MYELOMA CELL**-GROWTH IN SERUM-REDUCED AND **SERUM-FREE** MEDIA
AUTHOR: KOVAR J
CORPORATE SOURCE: INST MOLEC GENET, PRAGUE, CZECHOSLOVAKIA
COUNTRY OF AUTHOR: CZECHOSLOVAKIA
SOURCE: CELL AND TISSUE KINETICS, (1984) Vol. 17, No. 3, pp. 284-284.
ISSN: 0008-8730.
PUBLISHER: BLACKWELL SCIENCE LTD, OSNEY MEAD, OXFORD, OXON, ENGLAND OX2 0EL.
DOCUMENT TYPE: Conference; Journal
FILE SEGMENT: LIFE
LANGUAGE: English
REFERENCE COUNT: 0
ENTRY DATE: Entered STN: 1994
Last Updated on STN: 1994

ANSWER 38 OF 43 MEDLINE on STN DUPLICATE 9
ACCESSION NUMBER: 84210808 MEDLINE <<LOGINID::20070129>>
DOCUMENT NUMBER: PubMed ID: 6724641
TITLE: **Serum-free** medium for hybridoma and parental **myeloma cell** cultivation: a novel composition of growth-supporting substances.
AUTHOR: Kovar J; Franek F
SOURCE: Immunology letters, (1984) Vol. 7, No. 6, pp. 339-45. Journal code: 7910006. ISSN: 0165-2478.
PUB. COUNTRY: Netherlands
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 198407
ENTRY DATE: Entered STN: 20 Mar 1990
Last Updated on STN: 20 Mar 1990
Entered Medline: 18 Jul 1984

AB Serum-free chemically defined medium for hybridoma and parental myeloma cultivation was developed on the basis of testing of individual substances supporting hybridoma growth under serum-free conditions. Optimized concentrations of transferrin, insulin, ethanolamine, linoleic acid, serum albumin, ascorbic acid, hydrocortisone, and trace elements could substitute serum. Developed serum-free hybridoma (SFH) medium differs from analogous previously described media mainly by a more complete combination of growth-supporting supplements and by the presence of ascorbic acid and hydrocortisone. Growth comparable with that in the medium supplemented with 10% bovine serum was achieved with four hybridomas and two myelomas. SFH medium was also suitable for long-term cultivation of hybridomas without cessation of monoclonal antibody production. Growth potency and the specific growth requirements of hybridomas in serum-free medium are, to a large degree, determined by parental myeloma.

ANSWER 35 OF 43 LIFESCI COPYRIGHT 2007 CSA on STN
ACCESSION NUMBER: 86:45740 LIFESCI <<LOGINID::20070129>>
TITLE: **Serum-free** medium for hybridoma and parental **myeloma cell** cultivation. IMMUNOCHEMICAL TECHNIQUES. PART I. HYBRIDOMA TECHNOLOGY AND MONOCLONAL ANTIBODIES.
AUTHOR: Kovar, J.; Franek, F.; Langone, J.L. [editor]; van Vunakis, H. [editor]
CORPORATE SOURCE: Dep. Membr. Biochem., Inst. Mol. Genet., Czechoslovak Acad. Sci., CS-142 20 Prague 4, Czechoslovakia
SOURCE: METHODS ENZYMOL., (1986) pp. 277-292.
DOCUMENT TYPE: Book
FILE SEGMENT: F; W
LANGUAGE: English

AB Many cell lines including hybridomas and parental myelomas can grow in serum-free media supplemented with hormones, transferrin, lipids, trace elements. and other factors. Several laboratories have reported cultivation of hybridomas in serum-free chemically defined media developed for this purpose. Iscove's medium was the first serum-free medium successfully used for hybridoma cultivation. SFH (serum-free hybridoma) medium is characterized by the largest set of supplements which are known to support hybridoma growth under serum-free conditions.

ANSWER 32 OF 43 BIOTECHDS COPYRIGHT 2007 THE THOMSON CORP. on STN
ACCESSION NUMBER: 1987-03362 BIOTECHDS <<LOGINID::20070129>>
TITLE: New human plasma myeloma cell line NCI-H929; can be established in continuous culture; useful as fusion partner for hybridoma production
PATENT ASSIGNEE: U.S.Dept.Health-Human-Serv.
PATENT INFO: US 6854493 30 Sep 1986
APPLICATION INFO: US 1986-854493 22 Apr 1986
PRIORITY INFO: US 1986-854493 22 Apr 1986
DOCUMENT TYPE: Patent
LANGUAGE: English
OTHER SOURCE: WPI: 1987-007130 [01]
AN 1987-03362 BIOTECHDS <<LOGINID::20070129>>
AB The human plasma myeloma cell line NCI-H929 has rearranged alpha and kappa genes and secretes large amounts of IgAk (over 80 ug/million cells/24 hr). The cell line was prepared from the malignant effusion of a patient with IgAk **myeloma**. **Cells** were cultured on **serum-free** medium. The cell population doubled in about 50 hr. The cells were irregularly shaped, floating cells (singly or in small clusters) with many giant forms, some multinucleated, being present. The cell line has been deposited as ATCC CRL 9068. Cells initially isolated are mostly aneuploid but after culture cells of DNA index 2.3 become predominant. After 87 days of culture, about 80% of metaphases are nearly tetraploid. These tetraploid cells contain 6 copies of chromosomes 1 and 8; at least 4 of the chromosome 8 copies have additional material attached to the long arm. All copies of chromosome 1 are abnormal. The myeloma cell line can be established in continuous in vitro culture, and is potentially useful as a fusion partner in hybridoma construction. (34pp)

ANSWER 31 OF 43 HCAPLUS COPYRIGHT 2007 ACS on STN
ACCESSION NUMBER: 1989:73823 HCAPLUS <<LOGINID::20070129>>
DOCUMENT NUMBER: 110:73823
TITLE: Reducing costs upfront: two methods for adapting hybridoma cells to an inexpensive, chemically defined serum-free medium
AUTHOR(S): Brown, Bruce L.
CORPORATE SOURCE: Hazleton Biotechnol. Co., Vienna, VA, USA
SOURCE: Bioprocess Technology (1987), 2(Commer. Prod. Monoclonal Antibodies), 35-48
CODEN: BPTEEP; ISSN: 0888-7470
DOCUMENT TYPE: Journal
LANGUAGE: English
AB An inexpensive chemical defined medium that supports the growth and maintenance of a mouse myeloma cell line suitable for use as a fusion partner is described. Also, a method for adapting a monoclonal antibody-producing cell line growing in a serum-containing medium to serum-free medium with little or no loss of antibody secretion is described.

ANSWER 28 OF 43 HCAPLUS COPYRIGHT 2007 ACS on STN
ACCESSION NUMBER: 1988:527350 HCAPLUS <<LOGINID::20070129>>
DOCUMENT NUMBER: 109:127350
TITLE: **Myeloma cell** line adapted to **serum-free** medium supplemented with transferrin and its use in hybridoma formation and monoclonal antibody production
INVENTOR(S): Brown, Bruce L.
PATENT ASSIGNEE(S): Hazleton Biotechnologies, Inc., USA
SOURCE: U.S., 5 pp.
CODEN: USXXAM
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
US 4757018	A	19880712	US 1985-700199	19850211
PRIORITY APPLN. INFO.:			US 1985-700199	19850211

AB A myeloma cell line is provided which is adapted for growth in a serum-free medium in which human transferrin is the sole protein source. These cells may be fused to immunocytes for monoclonal antibody production Myeloma cells were initially cultured in serum-supplemented media. Over 6 wk, the cells were adapted to serum-free growth conditions by serial reduction of serum and supplementation of the medium with 50 μg human transferrin/mL. After several passages in the final medium (L-glutamine 292.0, Na pyruvate 110.0, transferrin 3.3, and optionally penicillin/streptomycin 100.0 mg/L), the cell line was cloned by limited cell dilution Spleen cells from mice immunized with thyroglobulin or lactogen were fused with the adapted cell line in the serum-free medium. The resultant hybridomas produced desired monoclonal antibodies.

ANSWER 27 OF 43 HCAPLUS COPYRIGHT 2007 ACS on STN
ACCESSION NUMBER: 1988:453212 HCAPLUS <<LOGINID::20070129>>
DOCUMENT NUMBER: 109:53212
TITLE: Manufacture of recombinant human tissue plasminogen activator with improved homogeneity using myeloma cells
INVENTOR(S): Gillies, Stephen D.
PATENT ASSIGNEE(S): Damon Biotech, Inc., USA
SOURCE: PCT Int. Appl., 58 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 8800242	A1	19880114	WO 1987-US1569	19870625
W: AU, DK, FI, JP, NO				
RW: AT, BE, CH, DE, FR, GB, IT, LU, NL, SE				
AU 8776960	A	19880129	AU 1987-76960	19870625
EP 272315	A1	19880629	EP 1987-904614	19870625
R: AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE				
JP 63502726	T	19881013	JP 1987-504281	19870625
DK 8800968	A	19880422	DK 1988-968	19880224
FI 8800891	A	19880225	FI 1988-891	19880225
NO 8800831	A	19880425	NO 1988-831	19880225
PRIORITY APPLN. INFO.:			US 1986-879038 A	19860626
			WO 1987-US1569 A	19870625

AB Recombinant human tissue plasminogen activator (tPA) with improved homogeneity and biol. activity is manufactured with transformed **myeloma cells** grown in **serum-free**, ε-aminocaproic acid-containing medium. Myeloma cell line J558L was transformed with pEMp1-tPA, which contains Bowes melanoma tPA cDNA. Encapsulated cells were grown as described and tPA was purified using controlled-pore glass and immobilized anti-tPA monoclonal antibody. The resulting tPA compns., which were typically >90% single-chain, mature tPA, were extensively tested in vitro and in animal models (rabbits, dogs). The recombinant tPA had an increased kcat in the presence of fibrin.

ANSWER 6 OF 43 HCAPLUS COPYRIGHT 2007 ACS on STN
ACCESSION NUMBER: 1996:290368 HCAPLUS <<LOGINID::20070129>>
DOCUMENT NUMBER: 124:341059
TITLE: Serum-free animal tissue culture medium for mass production of proteins
INVENTOR(S): Sawada, Hidekazu; Ito, Takashi; Maejima, Kazutaka
PATENT ASSIGNEE(S): Takeda Chemical Industries Ltd, Japan
SOURCE: Jpn. Kokai Tokkyo Koho, 13 pp.
CODEN: JKXXAF
DOCUMENT TYPE: Patent
LANGUAGE: Japanese
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
JP 08070859	A	19960319	JP 1995-150683	19950616
PRIORITY APPLN. INFO.:			JP 1995-150683 A	19950616
			JP 1994-144172	19940627

AB A serum-free animal tissue culture medium composition containing inorg. or organic Fe compds., cyclodextrin, non-ionic surfactants, and, optionally, insulin, ethanolamine, and selenites is provided. The medium may supplemented with dexamethasone, protein hydrolyzates, and amino acids. Production of t-gD-IL-2, a fusion protein of herpes simplex virus (HSV) type 1 glycoprotein D (t-gD) and human interleukin-2 (IL-2), by cultivating mouse myeloma cell strain Sp2/0-22-32-34 in this medium was demonstrated.

ANSWER 30 OF 43 BIOTECHDS COPYRIGHT 2007 THE THOMSON CORP. on STN
ACCESSION NUMBER: 1988-00440 BIOTECHDS <<LOGINID::20070129>>
TITLE: Production of monoclonal antibodies to peanut-mottle virus and their use in enzyme-linked immunosorbent assay and dot-immunobinding assay;
monoclonal antibody preparation; hybridoma construction
AUTHOR: Sherwood J L; Sanborn M R; Keyser G C
LOCATION: Department of Plant Pathology, Oklahoma State University, Stillwater 74078, U.S.A.
SOURCE: Phytopathology; (1987) 77, 8, 1158-61
CODEN: PHYTAJ
DOCUMENT TYPE: Journal
LANGUAGE: English
AN 1988-00440 BIOTECHDS <<LOGINID::20070129>>
AB Peanut-mottle virus (Pmv) infects a number of legume species. A monoclonal antibody (MAb) specific for it was used to develop an ELISA and dot-immunobinding assay for the detection of Pmv in foliar tissue. BALB/c mice were injected i.m. with 250 ug of the Pmv-OK isolate emulsified with Freund's complete adjuvant 3 times at wk intervals and then given 250 ug of virus in distilled water. Spleens were rinsed in a balanced salt solution, pressed through a mesh and cells suspended in serum-free RPMI 1640. These were fused (1:1) with mouse **myeloma cell** line P3X63Ag8.653 in **serum-free** RPMI 1640 with 35% PEG-1000 at pH 7.8. The next day cells were given HAT medium. Ascitic fluid containing MAb was produced in pristane-primed BALB/c mice by injecting 1 million hybridoma cells i.p. and collecting the fluid after 2-3 wk. (21 ref)

ANSWER 49 OF 67 HCAPLUS COPYRIGHT 2007 ACS on STN
ACCESSION NUMBER: 1993:211004 HCAPLUS <<LOGINID::20070129>>
DOCUMENT NUMBER: 118:211004
TITLE: Monoclonal ligand binding site related anti-idiotypic antibodies elicited with a polyclonal kinin antibody
AUTHOR(S): Odya, Charles E.; Yapa, Ramani; Soltani-Tehrani, Bahram; Carlin, Robert J.; **Lee, Che Hung**
CORPORATE SOURCE: Sch. Med., Indiana Univ., Bloomington, IN, 47405, USA
SOURCE: Hybridoma (1993), 12(1), 45-53
CODEN: HYBRDY; ISSN: 0272-457X
DOCUMENT TYPE: Journal
LANGUAGE: English

AB Splenocytes from mice immunized with homogeneous, polyclonal, rabbit kinin antibody (BK21) were fused using PEG with the mouse myeloma cell line **SP2/0**. Eleven monoclonal antibodies, whose binding to BK21 could be inhibited by bradykinin, were obtained from 3 fusions. All of these anti-idiotypic antibodies were of the IgG1,κ isotype, except for one, which was an IgG2a,κ. An IgM,κ auto-anti-idiotypic antibody, reactive with BK21 was obtained from a fusion of **SP2/0** cells and splenocytes from a mouse immunized with bradykinin conjugated with carbodiimide to keyhole limpet hemocyanin. Bradykinin could completely inhibit the binding of all of the anti-idiotypic antibodies to BK21 in an ELISA. This result is consistent with the anti-idiotypic antibodies being reactive with the ligand binding sites of BK21. It was possible to sep. the antiidiotypic antibodies into 2 groups. The first group, 10 of the 12 antibodies tested, was more sensitive to inhibition by bradykinin than the second group and was not readily inhibited by des-Arg9-bradykinin. The second group was about 7 times more sensitive to inhibition by des-Arg9-bradykinin than by bradykinin.

ANSWER 13 OF 43 BIOTECHDS COPYRIGHT 2007 THE THOMSON CORP. on STN
ACCESSION NUMBER: 1991-07066 BIOTECHDS <<LOGINID::20070129>>
TITLE: Efficient expression of recombinant antibodies in mammalian cells;
human, mouse chimeric antibody, monoclonal antibody; DNA amplification, expression in CHO or **myeloma cell** culture; **serum-free** culture medium; antibody engineering (conference abstract)
AUTHOR: Roberts G; Bebbington C R; Renner G; Gofton C M; Thomson S; McCormack M
CORPORATE SOURCE: Celltech
LOCATION: Celltech Limited, 216 Bath Road, Slough, Berkshire, UK.
SOURCE: J.Cell.Biochem.; (1991) Suppl.15E, 122
CODEN: JCEBD5
DOCUMENT TYPE: Journal
LANGUAGE: English
AN 1991-07066 BIOTECHDS <<LOGINID::20070129>>
AB The clinical use of humanized monoclonal antibodies (MAbs) will necessitate the efficient production of recombinant proteins from mammal cells. This is frequently achieved by repeated gene amplification of vector sequences integrated into the host cell chromosomes e.g. dihydrofolate-reductase (EC-1.5.1.3) gene amplification selected using methotrexate in dhfr- Chinese hamster ovary (CHO) cell culture. Glutamine-synthetase (GS, EC-6.3.1.2) is an alternative amplifiable selectable marker gene which can be used in a variety of cell types. Using the GS system, the efficient expression of immunoglobulin cDNAs under the control of strong viral promoters after only a single round of gene amplification can be achieved. The expression of a mouse-human chimeric antibody in either CHO or myeloma cells results in production of 250 mg/l MAbs in suspension cell culture in serum-free culture medium. The GS system facilitates large-scale recombinant MAb production economically from a variety of different mammal cell cultures. (0 ref)